Agricultural Research Institute
PUSA

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

CONTENTS OF VOLUME 138

No. 1, MARCH, 1941

Contents

Contents

Letters to the Editors

No. 2, APRIL, 1941

Contents

Contents

STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

II. THE BIOASSAY OF HEMORRHAGIC CONCENTRATES BY FOLLOWING THE PROTHROMBIN LEVEL IN THE PLASMA OF RABBIT BLOOD*

By HAROLD A. CAMPBELL, WILLIAM K. SMITH, WILLARD L. ROBERTS, AND KARL PAUL LINK

(*From the Departments of Biochemistry and Genetics, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison*)

The work of Roderick (1–3) had shown that in the hemorrhagic sweet clover disease of the bovine the prothrombin of the blood was the component affected. Quick (4) confirmed this and reported some quantitative prothrombin determinations on the plasma of rabbits fed a diet of spoiled sweet clover[1] and ground oats.

In the early stages (1935–37) of our studies (5–7) we employed the method developed by Quick for the bioassays (8). An extensive study of the prothrombin level (or activity) in normal and pathic[2] rabbit plasma led us to develop a prothrombin assay better suited to our experimental purposes (7).[3]

---

* Cooperative studies with the Division of Forage Crops and Diseases, United States Department of Agriculture, Washington, and the Wisconsin Agricultural Experiment Station, and published with the approval of the Director of the Wisconsin Agricultural Experiment Station, paper No. 268, Genetics Department. Personnel and supply assistance in part through the Natural Science Research Project No. 65-1-53-2349 of the Federal Works Progress Administration, Madison.

[1] Obtained from our supply of experimentally produced spoiled hay in October, 1936.

[2] By pathic plasma we mean plasma in which the prothrombin level (or activity) has been altered from the normal by the hemorrhagic agent in spoiled sweet clover hay or concentrates therefrom. See the paper by Link (9) on the rôle of genetics in etiological pathology for the derivation of the term pathic and its preferential use to pathological in this instance (p. 134).

[3] Campbell, H. A., Overman, R. S., and Link, K. P., Studies on the physiology of rabbit blood (1938), unpublished.

1

The method developed by us is based in principle on the procedure of Quick *et al.* (8) which involves the determination, under standard conditions, *of the time* required for the clotting of recalcified plasma in the presence of an excess of added thromboplastin. This is the time required for the following consecutive reactions: (1) the interaction between prothrombin, calcium, and thromboplastin to form thrombin; (2) reaction of thrombin with fibrinogen to form fibrin (the clot).

It has been pointed out by Smith and coworkers (10, 11) that the time involved in the first reaction is dependent on the prothrombin content as well as other factors. They have also indicated that in the production of a clot in normal plasma only a small amount of the prothrombin present is converted to thrombin. To circumvent this issue the time involved in the *second* phase of the reaction was regarded by them as a measure of the prothrombin content.

In conjunction with our extensive control studies on normal and pathic rabbit plasma (5–7), we have made numerous observations[3] that have a bearing on the controversial topic of blood coagulation. However, no useful purpose would be served by introducing those observations at this time. Howell (12) and Eagle (13) have recently published critical and comprehensive reviews on the subject. If the views of Smith *et al.* are accepted, it would appear that their titration method is on a firmer theoretical basis than the method of Quick, since it has been established that the clotting time is proportional to the thrombin content. But for the work described in the previous paper (14) and additional studies to follow, the reproducibility of relative clotting time values is of greater importance than their absolute magnitude. Since the method of Smith *et al.* requires the purification of the various components involved in the clotting mechanism, variables other than prothrombin might possibly thereby be admitted. In addition, the labor demanded in the preparation of the components and the time required for the making of serial titrations put limitations on the number of animals that can be tested. Accordingly, the method of Smith and coworkers was, after due consideration, not adopted for our work.

We have introduced several important modifications in Quick's prothrombin method which have greatly increased the reproduc-

ibility and decreased the minimum detectable change in pro-
thrombin. The modifications are: (1) The method of prepar-
ing the thromboplastin[4] used in the clotting process and the order
of combining the reagents have been changed. (2) Only appro-
priately diluted plasma is used. This permits the detection of
smaller changes in the prothrombin activity. (3) The clotting
times are evaluated through the use of a log-log dilution curve
constructed for the particular rabbit under assay. (4) The
assay has been put on a *strictly differential basis*, which eliminates
the necessity of obtaining absolute values, since the pathic plasma
is compared directly with plasma from the same rabbit in the
normal state.

In brief, we measure in each test rabbit the change in the
prothrombin level (or activity), within a certain range, produced
by feeding spoiled sweet clover hay samples or concentrates
prepared therefrom. These modifications will be discussed indi-
vidually as they appear below. Their adoption has enabled us
in the absence of a chemical test to make uninterrupted progress
in the concentration of the hemorrhagic agent, and have led to
concentrates free from N, S, and P with a potency approximately
20,000 times greater than the original hay.

Prothrombin Determination

The prothrombin determination, like any quantitative method
based on empirical rather than stoichiometric relationships, can
only give reliable and reproducible results by the adherence to
rigidly standardized manipulative conditions. This is especially
the case when one is dealing with a process as sensitive and vari-
able as the phenomenon of blood clotting. If the procedure
given below is followed carefully, reproducible results will be
obtained.

Reagents and Solutions—The reagents and solutions, (1) sodium
oxalate, (2) normal physiological saline solution, and (3) calcium

---

[4] In the prothrombin determination that Almquist and Klose (15) de-
veloped for the assay of the antihemorrhagic vitamin, these authors have
also found it advisable to alter Quick's method of preparing the thrombo-
plastin emulsion. Sauter, Kark, and Taylor (16) have recently also re-
ported a method for the preparation of a more stable thromboplastin for
use in Quick's test.

chloride, are made up as directed by Quick (8). The thromboplastin[4] is prepared as follows:

Thromboplastin.   Preparation of Dry Rabbit Brain Tissue—A rabbit is decapitated with a sharp pair of tin snips. The brain tissue and the readily available medulla are removed. The brain tissue is freed from blood vessels by removing the free pia mater under a stream of water. It is then ground to a smooth paste in a mortar, spread out on a watch-glass, and dried in a vacuum desiccator containing calcium chloride. The air is evacuated until a large foamy mass is obtained, whereupon the desiccator is maintained at 38–40° for 24 hours. *These operations are all executed as rapidly as possible.* The resulting dry tissue is finally broken up to a fine powder and stored in a glass-stoppered bottle at 0°. Preparations made by this procedure have retained their activity over a period of several months when stored at 0°. Obviously there are slight differences in the activity of individual thromboplastin preparations.

Preparation of Thromboplastin Emulsion—To 0.10 gm. of the dried brain tissue (equivalent to approximately 0.5 gm. of fresh brain) 5 cc. of saline solution are added. The solution is then stirred until a uniform suspension is obtained. The suspension is heated (with constant stirring) at 54–55° in a water bath for 10 minutes to destroy the prothrombin activity. The suspension is then cooled to 25–26°. To the cooled suspension 5.0 cc. of the 0.025 M calcium chloride solution are added. After being stirred for 4 minutes, the mixture is centrifuged 4 minutes at 1700 R.P.M. The spindle of the centrifuge is brought to a standstill slowly so as to avoid resuspending the flocculent precipitate. The clear or slightly turbid supernatant solution is removed with a pipette.

Preparation of Plasma to Be Assayed—After the marginal surface of the rabbit's ear has been shaved, xylene is dropped along the medial edge to stimulate circulation. The blood syringe is rinsed with 0.1 M oxalate solution and 0.2 cc. of oxalate is drawn into the cylinder. From the dilated marginal vein 1.8 cc. of blood are drawn, and mixed quickly by rotating the syringe. The needle is removed from the syringe and the oxalated blood is forced slowly into a 75 × 10 mm. test-tube. The organized bodies are removed by centrifuging at 1700 R.P.M. The clear plasma is then transferred to another test-tube with a pipette. If the

proper manipulative technique is employed in drawing the blood sample, no evidence of clots should be detected. The clotting time of the plasma may be determined immediately, but in the assay of spoiled sweet clover hays and hemorrhagic concentrates prepared therefrom the plasma is stored at 0° and is compared directly with the plasma obtained after feeding an active fraction.

Clotting Time of Normal Plasma—Into two 75 × 10 mm. test-tubes, 0.1 cc. of plasma is added with a serological pipette graduated in 0.001 cc. This plasma is now appropriately diluted with saline solution. To one test-tube containing normal plasma 0.7 cc. of saline is added to give a plasma concentration of 12.5 per cent. To the other test-tube 1.1 cc. of saline are added to give a plasma concentration of 8.34 per cent. The diluted plasma is mixed thoroughly[5] and placed in the constant temperature water bath at 37°.

Into 100 × 12 mm. test-tubes, 0.2 cc. of the thromboplastin-calcium chloride solution is transferred with a 0.2 cc. micro blood sugar pipette. These tubes are placed in a rack beside the diluted plasma samples in the constant temperature bath. As soon as the contents of the tubes have reached the bath temperature, the clotting time of the plasma is determined for the plasma concentrations of 12.5 and 8.34 per cent.

After the contents have been mixed, 0.1 cc. of the diluted plasma is transferred with a 0.1 cc. micro blood sugar pipette to a tube containing 0.2 cc. of the thromboplastin-calcium chloride solution. The diluted plasma is quickly blown from the pipette. At the same time the stop-watch is started.[6] The tube is tapped sharply to mix the solutions. This insures a uniform initiation of the clotting process throughout the solution. A small stirrer made of No. 22 nichrome wire is now introduced. During the clotting process the solution is stirred at such a rate that the stirrer paddle sweeps across the test-tube from one side to the other two times per second. The end-point (formation of the clot) is that point

[5] The plasma is conveniently mixed by firmly holding the test-tube near the top with the thumb and index finger, and striking the lower end sharply with glancing blows from the index finger of the other hand. This accomplishes a thorough agitation without the possible contamination introduced by the use of corks or stirring rods.

[6] The stop-watch may be conveniently operated by a foot treadle.

at which the fibrin clot is sufficiently stable to be drawn to one side by the stirrer, thus bringing into view a clear area. The clot is usually somewhat turbid, since the calcium oxalate formed upon calcifying the oxalated plasma is enmeshed in the clot. The formation of fibrils, which impart a viscous appearance to the solution before the clot forms, can be disregarded.

The results realized with the above technique on a given plasma are characteristic and reproducible. With diluted plasma (12.5 per cent concentration) having a clotting time of 30 seconds, the variations between duplicate determinations will be 1 second or less.[7]

Clotting Time of Pathic Plasma—Immediately after the clotting time of the stored normal plasma has been measured, the clotting time of the pathic plasma from the same rabbit is determined in exactly the same manner. Two 0.1 cc. samples of plasma are pipetted out. To one is added 0.7 cc. of saline solution and the clotting time measured. With this clotting time as a guide of the clotting power of the pathic plasma, the second 0.1 cc. sample of plasma is diluted with an appropriate amount of saline solution so that its clotting time will fall between that of the normal plasma of 12.5 and 8.34 per cent concentration already determined. The choice of a plasma concentration so that the clotting time falls between that of the normal plasma of 12.5 and 8.34 per cent concentration is facilitated by reference to a plasma dilution curve (Fig. 1) or to the log-log curve (Fig. 3) prepared for the rabbit under assay. We have found that, when the pathic plasma is compared with the normal plasma, concentrations of pathic plasma higher than 25 per cent should not be used. When the clotting time of the pathic plasma falls outside the clotting times obtained for the 12.5 and 8.34 per cent concentrations of the normal plasma, it is advisable to use another dilution of the normal plasma. We select a dilution of the normal plasma which will give the same clotting time obtained with the pathic plasma at a concentration of 25 per cent. Through experience with the method one is able to make the appropriate choice of concentration by mere inspection of the normal clotting times and the clotting time of the pathic plasma of 12.5 per cent concentration (see the example below).

---

[7] When undiluted plasma is used, the clotting time in the range of 10 seconds is reproducible within a few tenths of a second.

Advantages in Manipulation Realized in Our Modification of Quick's Prothrombin Determination—The manipulative art described above is based upon extensive control experimentation that cannot be included here (6, 7).[3]   It should be noted that in the original prothrombin method of Quick the calcium chloride solution is added to the plasma separately.   It was found advisable in the development of our modification of Quick's procedure to add the calcium chloride and the thromboplastin at one time. One possible error in pipetting solutions is thereby avoided and, since the transfer of solution is made to an empty test-tube, there is no opportunity for contamination.   Freedom from contamination of stock solutions may be realized with the original Quick procedure if a clean pipette be used for each addition of calcium chloride.   However, if this is not done, even though extreme care be exercised to avoid touching the wall of the test-tube containing the plasma and thromboplastin, sufficient splattering may take place to induce contamination of the stock solutions when a large number of determinations are being made.   If calcium chloride is added to the milky and somewhat viscous thromboplastin used by Quick, some inactive protein is coagulated which can be removed by centrifuging.   The perfectly clear solution obtained in this manner is just as active as the milky suspension.

It has been known for many years that the calcium content has a marked effect on the clotting time of blood.   Stewart and Pohle (17) have recently shown that the optimum calcium content is not the same for all individuals (man).   The variations in the optimum calcium content for the individual animals might affect the *absolute* values of the clotting times observed.   Since our assay method is strictly a differential method, these variations in absolute values will not influence the reliability of the assay.   If absolute or minimum values for the clotting time are sought, they can be realized by making serial determinations with various concentrations of calcium chloride as suggested by Stewart and Pohle (17).

Effect of Plasma Dilution on Clotting Time

Fig. 1 indicates that our method gives a dilution curve of the same general form as that obtained by Quick.   The clotting time is only slightly changed until a concentration of about 25 per cent is reached.   It is also evident from our results, in confirmation of

Quick's findings, that plasma concentrations below 5 per cent give irregularly large increases in clotting time upon further dilution.

It was observed (6) that rabbit plasma differing but slightly in prothrombin content (mildly pathic plasma) could not be distinguished from normal plasma by comparing the clotting times of the undiluted plasma. However, after appropriate dilutions were made, such plasmas could be easily distinguished by the difference in clotting time. From a careful consideration of Fig. 1 it is evident that this is to be expected, since the clotting
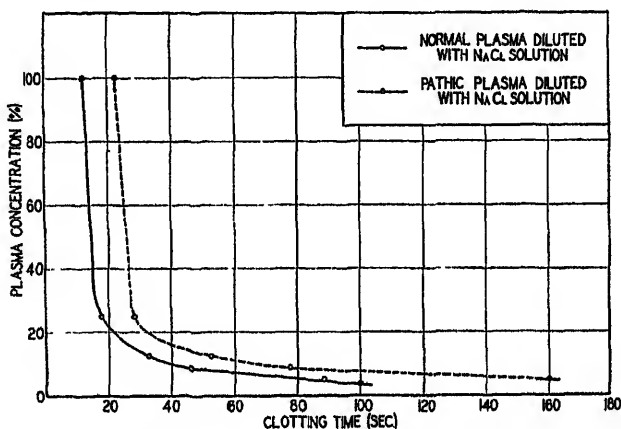
Fig. 1. The effect of plasma dilution on the clotting time as measured in the prothrombin assay.

time is influenced only slightly by small differences' in the plasma concentrations, when the plasma concentration is high.

This curve also indicates that the most reliable prothrombin values are obtained when the plasma concentration lies between the limits of 5 to 25 per cent. It is apparent that small and unavoidable errors in plasma concentration and in clotting time will have a minimum effect in this range. But the same does not hold with undiluted plasma when there is very little change in clotting time with variations in plasma concentration or prothrombin content. Likewise the accumulative errors become significant with very dilute plasma, since slight changes in concentration give rise to large and variable changes in clotting time.

By comparing a dilution of pathic plasma having a clotting time equal to that of plasma from the same rabbit in the normal state (within the plasma concentration range 12.5 to 8.34 per cent), we are able to detect much smaller changes in prothrombin than would otherwise be possible, and have also greatly diminished the adverse effect of antithrombins. The work of Eagle has shown that the effect of antithrombins is greatly decreased by dilution (18). These considerations form the basis for using the plasma concentration range of 12.5 to 8.34 per cent (one-eighth and one-twelfth plasma).

Use of Log-Log Curves to Facilitate Interpolation between Clotting Times of Known Concentrations of Plasma

The dilution curves of plasmas obtained from a number of normal rabbits are not identical. The clotting time of normal rabbit plasma of 12.5 per cent concentration varies between the values of 20 and 40 seconds. However, for any given rabbit this clotting time remains relatively constant. It is therefore evident that, in order to obtain accurate results, the average or median dilution curve for a number of rabbits should not be used to interpret the clotting time values of an assay rabbit, as is done in Quick's method, but a dilution curve must be constructed for the individual rabbit under assay. The preparation of dilution curves of the type shown in Fig. 1 would involve a large number of experimentally determined points for each assay rabbit and be impractical owing to the time involved. If the log of the clotting time is plotted against the log of the plasma concentration, the resulting curve falls on a straight line[8] over the range of concentrations of 5 (log 0.7) to 20 per cent (log 1.3) in Fig. 2. Since the dilution curve plotted in this manner falls on a straight line, the clotting time of the two concentrations of the normal plasma (12.5 and 8.34 per cent) suffices to establish this curve. The concentration of normal plasma that would have the same clotting time as the diluted pathic plasma is determined from this log-log curve. This is done by noting the ordinate of a point on the curve representing a clotting time equal to that of the pathic plasma. The antilogarithm of this ordinate is the concentration

[8] Plotting on a log-log graph paper will also result in a straight line curve.

of normal plasma which would have the same clotting time as that of the diluted pathic plasma. The extent of the diminution in the prothrombin activity of the rabbit plasma is expressed by the relative clotting index, which is the ratio of the concentration
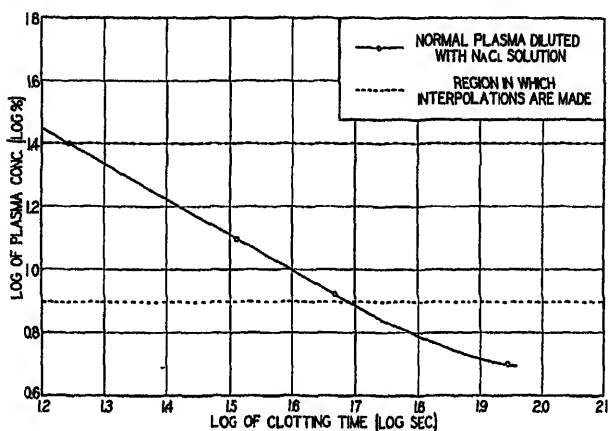
Fig. 2. Log-log dilution curve of rabbit plasma

TABLE I

Clotting (or Prothrombin) Time in Seconds of Normal and Pathic Plasma at Different Concentrations, before and after Feeding 50 Gm. of Standard Hay Sample

Rabbit No.	Normal plasma		Pathic plasma	
	12 per cent	8 per cent	12 per cent	8 per cent
S-51	29	36	34	56
S-52	29	40	42	58
S-53	25	33	34	50
S-82	27	35	28	59
S-77	24	31	50	55
S-79	25	35	63	75

of the normal plasma, in the concentration range of 12.5 to 8.34 per cent, to the concentration of the pathic plasma which gives the same clotting time. Thus the pathic plasma is evaluated by comparison with plasma taken from the *same rabbit* in the normal state. Some representative clotting times (in seconds) of normal

and pathic plasma at different concentrations before and after feeding 50 gm. of the standard hay sample are given in Table I.

Stability of Rabbit Plasma

Originally we determined the prothrombin content of the plasma from the normal assay rabbit *immediately* after the blood was drawn. The rabbit was then fed a physiologically active fraction and the prothrombin content of the plasma again determined after a suitable period (38 to 48 hours). An extended
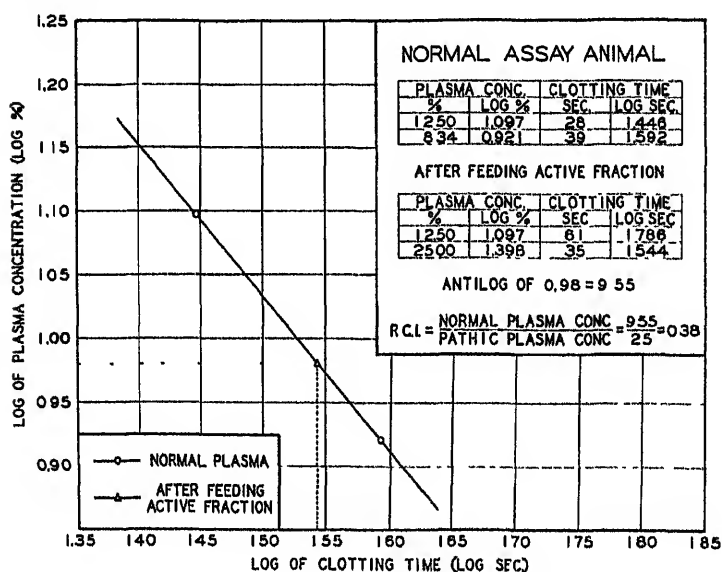
FIG. 3. Log-log dilution curve for the comparison of normal and pathic rabbit plasma.

study of the stability of rabbit plasma has made it possible to place the assay on a strictly differential basis.[3] This was realized by making the prothrombin estimation of both the normal and pathic plasma at the same time. The plasma from a normal assay rabbit is stored in a corked test-tube at about 0° until the pathic plasma is assayed (usually 2 days later). This permits a comparison of normal and pathic plasmas under identical conditions (Fig. 3).

The basis for this is in part the following: Samples of normal

plasma were stored at 0° and the prothrombin activity after varying periods of storage was compared with that of freshly prepared plasma. During a week of storage the variations in activity were not greater than the unavoidable daily variations of the normal rabbit and the activity of the thromboplastin emulsion.[9] After the normal plasma has been stored for more than a week, a measurable decrease in prothrombin activity can be detected. However, this decrease is small in comparison with the prothrombin depletion (or inactivation) that results from the feeding of moderately active fractions of the hemorrhagic agent.

In contrast to normal plasma, pathic plasma is not as stable. This difference in the relative stability of pathic (sweet clover plasma) and normal plasma has not been previously described by Roderick (1–3) and Quick (4) in their studies on the physiology of plasma obtained after feeding spoiled sweet clover hay. The prothrombin is apparently destroyed or inactivated *in vitro* upon standing. This decrease in prothrombin activity is accelerated at elevated temperatures. The stability of the pathic plasma is measured by incubating it at 37° for 4 to 8 hours. A rapid decrease in the prothrombin activity of pathic plasma takes place. Under the same conditions little or no change in the prothrombin values of normal plasma is noted.

Restoration of Prothrombin in Pathic Rabbits

The syndrome produced by feeding spoiled sweet clover hay or hemorrhagic concentrates prepared therefrom to cattle or rabbits (1, 2), if not allowed to proceed too far (or long), apparently does not result in permanent injury. If it be accepted that the liver is the site of prothrombin formation (10, 19–22), the continuity of its *synthesis* is either temporarily disturbed or there is a *qualitative* alteration in the prothrombin formed.[10] The pathic period is quite transient if the dosage is not too high and is followed by normal prothrombin restoration (see Fig. 4). When

---

[9] These variations are not detectable when undiluted plasma is used. With diluted plasma the method is sufficiently sensitive to detect these small but significant variations.

[10] Since early 1938 we have been cognizant of the fact that the hemorrhagic agent apparently does not inactivate the prothrombin of rabbit plasma *in vitro*.

less than 50 gm. of the standard spoiled hay or only mildly potent concentrates therefrom are fed, the pathic interval is reduced in severity and length, and the normal prothrombin level is restored in a proportionately reduced period. The maximum depletion of the prothrombin level, regardless of the dosage and the sensitivity of the rabbit, is usually realized under our conditions in 38 to 48 hours (Fig. 4).
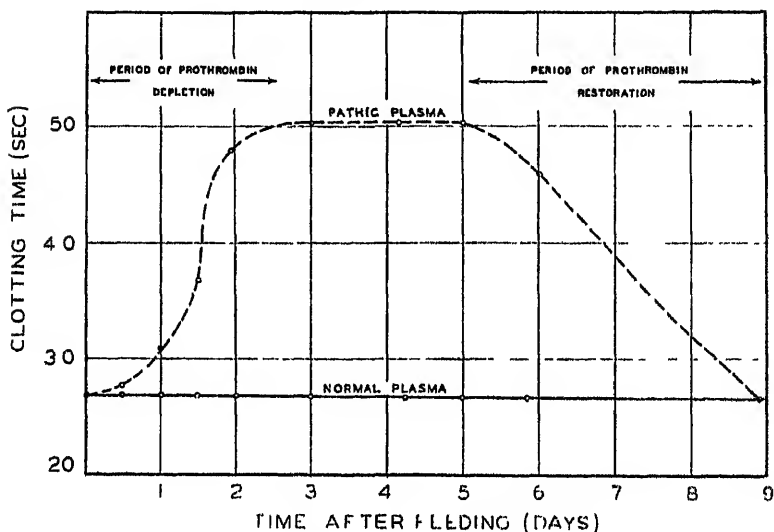
FIG. 4. Representative chart of prothrombin depletion resulting from the feeding of 50 gm. of standard spoiled sweet clover hay and subsequent restoration as revealed by the change in the clotting time (seconds) of pathic plasma.

Equally significant is the observation that the rabbit does not acquire immunity or increased susceptibility to the hemorrhagic agent. Roderick's extensive studies (2, 3) had established these cardinal facts with the bovine. This makes it possible to use the same assay rabbits repeatedly after allowance is made for a brief period of recovery.

We usually allow 12 days between assays with our standardized susceptible rabbits (see "Standardization of assay rabbits" below) to insure an ample margin of safety, although we have noted that the normal prothrombin level is usually restored within 4

to 6 days after the plateau of maximum depletion has been reached (Fig. 4).   Through judicious care we have been able to use the same rabbits throughout their normal cage life (4 years).   Some of them have been used for over 100 assays.   We have, of course, experienced some unavoidable losses through spontaneous hemorrhage due to misgaging the dosage of highly potent hemorrhagic concentrates, and to duels between animals which result in trauma shortly after assay.

If the assay rabbit is fed the stock grain mixture and alfalfa immediately after the plateau of prothrombin depletion is reached, the restoration to the normal prothrombin level appears to proceed more rapidly (19).   This phase of our work (dietary factors necessary for prothrombin formation) will be dealt with in a separate communication.   For the present it will suffice to state that a vitamin K concentrate prepared from *Medicago sativa* and the synthetic antihemorrhagic substance 2-methyl-1,4-naphthoquinone alone proved to be ineffective in either preventing the hemorrhagic action of spoiled sweet clover hay or concentrates prepared therefrom.   Likewise, it appears that the crude alfalfa concentrate and the quinone when fed alone do not materially hasten the restoration of the normal prothrombin levels in pathic rabbits.   It appears therefore that the sweet clover disease cannot be considered as strictly analogous to a vitamin K deficiency (13).

Standardization of Assay Rabbits

The problem of individual variation is usually met in bioassays by employing a statistically significant number of assay animals. In our assay method we have met the problem of biological variation (5, 7, 23)[3] by standardizing the *individual* rabbits.   As mentioned above this has been possible since it was established that the assay rabbit returns to the normal state rapidly without the development of immunity.

The standardization of the assay rabbit is carried out as follows: (1) The rabbit is given only water for 24 to 48 hours.   (2) A sample of blood is taken from the normal animal.   The organized bodies are removed by centrifuging and the plasma is stored at 0°. (3) The fasted rabbit is fed 50.0 gm. of spoiled sweet clover hay

of known physiological activity.[11] If 25¯ gm. of molasses are
added, the rabbits eat the ground hay more freely. The rabbit
should eat two-thirds of the hay during the first 8 hours and the
entire portion in 24 hours. (4) Between the 36th and the 42nd
hour after the start of the feeding of the physiologically active
material the blood sample is taken from the pathic animal. (5)
The clotting time of the pathic plasma is compared directly with
the clotting time of the plasma taken from the same rabbit in the
normal state.

The extent of change in the prothrombin level (or activity)
in the plasma is expressed by the relative clotting index. It is
apparent that a relative clotting index of 1.0 indicates that the
plasma prothrombin level is normal, while the plasma of pathic
rabbits will have a relative clotting index less than 1.0.

When a group of rabbits was standardized in the manner de-
scribed, it was found that there is a marked variation in the *sen-
sitivity* of the individuals to the hemorrhagic agent in spoiled sweet
clover hay (7). This phase of the problem will be treated sepa-
rately in Paper III (24). It is sufficient to say at this point that
individual rabbits vary in their physiological reaction from very
susceptible to very resistant to a given quantity of spoiled sweet
clover hay (50 gm.) and this variation is inherited as a Mendelian
character.[12] Since 1937 all bioassays required by our work on the
isolation of the hemorrhagic agent have been carried out with
standardized susceptible rabbits bred specifically for this purpose
(25). This resistance to 50 gm. of our spoiled hay is not an ab-
solute, but a relative resistance. The amount of spoiled sweet
clover hay used in the standardization will depend on the sus-

[11] In all of our work the same sample of spoiled sweet clover hay has
been used to standardize assay rabbits. Roderick (2) has shown that
physiologically active spoiled sweet clover hay retains its activity with
little or no loss over a period of several years. Our work confirms this
observation.

[12] These findings do not support the claims by Quick (4) who advanced
the belief that the diet previous to the time of testing determines the reac-
tion of a rabbit, and concluded that the incorporation of 5 per cent of de-
hydrated alfalfa meal with the hemorrhagic hay was sufficient to prevent
any demonstrable reduction of the prothrombin level or any other symp-
toms of the sweet clover disease (see also (25)).

ceptibility of the rabbits used for the assay and the physiological activity of the hay. It is preferable to choose that quantity of hay which will give a relative clotting index between 0.30 and 0.50. With the assay colony used in this study, 50 gm. of experimentally produced spoiled sweet clover hay are used to standardize the assay rabbits. The manner in which this standard relative clotting index is used in the assay of prothrombin-destroying concentrates follows.

Method of Assaying Prothrombin-Depleting Concentrates

The assay of prothrombin-depleting concentrates prepared from spoiled sweet clover hay has also been placed on a strictly differential basis. The rabbit is handled exactly as it was in the original standardization. The aliquot of active material fed is so chosen that the diminution in the prothrombin level (or activity) will be about equal to that noted in the standardization.[11] When hay is being assayed, it is fed with 25 gm. of molasses. Concentrates of moderate potency are dissolved in the appropriate volatile organic solvent and poured over 20.0 gm. of ground oats. After the solvent is evaporated, the physiologically active material suspended on the oats is fed to the assay rabbit. Highly potent concentrates are given in gelatin capsules. The rabbit is then fed 20.0 gm. of ground oats.

By comparing the clotting times before and after feeding the physiologically active fraction, the relative clotting index is determined for the pathic plasma. This ratio of the concentration of the normal plasma to the concentration of the pathic plasma has been discussed in connection with the standardization of assay animals. By comparing the relative clotting index after feeding a physiologically active concentrate with the relative clotting index at the time of standardization, the activity of the concentrate is evaluated in terms of the spoiled sweet clover hay used in the standardization of the rabbits.

When the diminution of the prothrombin level (or activity) is roughly equal to that realized in the standardization procedure, it may be considered that the physiological activity is the same as that of the standardization sample. It often happens in feeding fractions of unknown activity that the diminution of the prothrombin level is much smaller than, or greatly exceeds, that

noted in the standardization.   In such cases a qualitative estimation is usually sufficient, but if a quantitative appraisal is desired the assay should be repeated with an appropriate aliquot of the physiologically active material, so that the diminution in the prothrombin level is equal to that noted in the standardization. The quantitative relationship between physiological activity and diminution in prothrombin level has been established and will be dealt with separately.   For the present the following relationship will suffice.   If at the time of standardization the relative clotting index was 0.50, then one-half of the standard dose will give an

TABLE II

Clotting (or Prothrombin) Time in Representative Assay on Control and Assay Rabbit

Rabbit No.	Plasma concentration	Clotting (or prothrombin) time	
		Normal plasma	Plasma after feeding
	per cent	*sec.*	*sec.*
S-2	12	29	30
		29	31
S-2	8	44	44
		44	45
S-96	12	27	61
		28	60
S-96	8	39	
		39	
S-96	25		35
			34

index of about 0.75 and 2 times the standard dose will give an index of about 0.20.

In order to clarify some of the points discussed, an example showing the clotting time values obtained in a typical bioassay is given in Table II.   The control Rabbit S-2 received 20 gm. of ground oats, while the assay rabbit received a physiologically active fraction suspended on 20 gm. of ground oats.

The assay data include the clotting time of plasma obtained from control Rabbit S-2 to indicate the reproducibility of the clotting time under the conditions of the assay.   Since the pathic plasma is compared directly with the plasma taken from the same assay rabbit in the normal state, one can omit a control rabbit

after it has been established that the technique of taking blood samples and clotting times is faultless and completely reproducible.

The logarithms of the clotting time of the two concentrations of normal plasma are plotted against the logarithms of the plasma concentrations given in per cent. A straight line is drawn through these two points, thus establishing the dilution curve for Rabbit S-96 (see Fig. 3). On this curve the point corresponding to the clotting time of the pathic plasma is located (log 35 seconds = 1.544). The ordinate of this point is 0.98. The antilogarithm of the ordinate (0.98) is 9.55, which is the concentration of normal plasma that would give a clotting time equal to that of the 25 per cent pathic plasma; *i.e.*, a clotting time of 35 seconds. Thus the relative clotting index or the ratio of the concentration of normal plasma to the concentration of pathic plasma having the same clotting time is equal to 9.55/25.0 or 0.38. At the time of standardization with 50 gm. of spoiled sweet clover hay, Rabbit S-96 had a relative clotting index of 0.42. Thus the weight of concentrate under assay had a physiological activity equal to about 50 gm. of the spoiled sweet clover hay used for standardization.

The reliability of this assay method has been established in the last 3 years during which time thousands of complete bioassays of physiologically active fractions have been made in conjunction with our studies on the isolation of the hemorrhagic agent in the spoiled sweet clover hay, and by this assay (in the absence of a chemical test) we have been able to make consistent progress. It should be mentioned that the assay (with the same thromboplastin preparation) can also be applied to the plasma of the rat, the dog, and the bovine.

SUMMARY

1. A method for determining the prothrombin level in rabbit plasma, based on the procedure of Quick, has been developed to appraise accurately the relative prothrombin-inactivating powers of spoiled sweet clover hay and concentrates prepared therefrom.

2. The procedure for the standardization of the assay rabbits and the method of evaluating prothrombin-inhibiting concentrates are given in detail. Rabbits vary greatly in their susceptibility to the hemorrhagic agent in spoiled sweet clover hay; hence only *susceptible* rabbits should be employed.

3. The reagents and components (except the thromboplastin) used in the prothrombin determination are similar to those of Quick, but the order of combining them has been changed. This diminishes the possibility of errors through the contamination of the reagents. Details are given for the preparation of thromboplastin whose activity is readily maintained several months by storage at 0°.

4. Only plasma diluted to the concentration range 25 to 5 per cent is used. This permits the detection of smaller changes in the prothrombin activity, and simultaneously diminishes the adverse effects of antithrombins.

5. Plasma from a normal rabbit can be stored at 0° for several days without undergoing an appreciable change in the prothrombin activity. This permits a simultaneous comparison of normal plasma drawn from the same rabbit after prothrombin depletion under identical experimental conditions and eliminates the necessity of obtaining absolute prothrombin values. The plasma of rabbits whose prothrombin level has been diminished owing to the action of the hemorrhagic agent in spoiled sweet clover hay is less stable than the plasma of normal rabbits.

6. The clotting times are evaluated through the use of a log-log dilution curve constructed for each rabbit under assay. The ratio of the concentration of the plasma before feeding (in the concentration range of 12.5 to 8.34 per cent) to the concentration of plasma after feeding, which gives the same clotting time, is taken as a relative index of the amount of prothrombin inactivated.

7. The rabbit does not acquire immunity to the hemorrhagic agent in spoiled sweet clover hay, which makes it possible to use the assay rabbits repeatedly after a brief period for recovery is allowed.

BIBLIOGRAPHY

1. Roderick, L. M., *Am. J. Physiol.*, 96, 413 (1931).
2. Roderick, L. M., and Schalk, A. F., *North Dakota Agric. Exp Stat.*, *Bull. 250* (1931).
3. Roderick, L. M., *J. Am. Vet. Med. Assn.*, 74, 314 (1929).
4. Quick, A. J., *Am. J. Physiol.*, 118, 260 (1937).
5. Annual reports to the Director of the Wisconsin Agricultural Experiment Station, Madison (1935-40).
6. Roberts, W. L., Summaries of doctoral dissertations, 1936-37, Madison, 2, 204-206 (1938).
7. Campbell, H. A., Summaries of doctoral dissertations, 1939-40, Madison, 5, 155 (1940).
8. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sc.*, 190, 501 (1935).
9. Link, G. K. K., *Quart. Rev. Biol.*, 8, 127 (1932).
10. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 114, 667 (1936).
11. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *J. Exp. Med.*, 66, 801 (1937).
12. Howell, W. H., *Physiol. Rev.*, 15, 435 (1935).
13. Eagle, H., *Medicine*, 16, 95 (1937); also A symposium on the blood and blood-forming organs, Madison, 242-264 (1940).
14. Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *J. Biol. Chem.*, 136, 47 (1940).
15. Almquist, H. J., and Klose, A. A., *Biochem. J.*, 33, 1055 (1939).
16. Sauter, A. W., Kark, K., and Taylor, F. H. L., *Science*, 91, 532 (1940).
17. Stewart, J. K., and Pohle, F. J., *Proc. Soc. Exp. Biol. and Med.*, 39, 532 (1938).
18. Eagle, H., *J. Gen. Physiol.*, 18, 531, 547, 818 (1934-35).
19. Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 20, 194 (1940).
20. Warner, E. D., *J. Exp. Med.*, 68, 831 (1938).
21. Scanlon, G. H., Brinkhous, K. M., Warner, E. D., Smith, H. P., and Flynn, J. E., *J. Am. Med. Assn.*, 112, 189 (1939).
22. Andrus, W. DeW., Lord, J. W, and Kauer, J. T., *Science*, 91, 49 (1940).
23. Burn, J. H., Biological standardization, London and New York (1937).
24. Campbell, H. A., Smith, W. K., Overman, R. S., and Link, K. P., *J. Agric. Research*, in press (1941).
25. Smith, W. K., *Science*, 87, 419 (1938).

STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

IV. THE ISOLATION AND CRYSTALLIZATION OF THE HEMORRHAGIC AGENT*

By HAROLD A. CAMPBELL AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison)

In a previous paper (1) we presented an extraction scheme for the preparation of hemorrhagic concentrates with a prothrombin-reducing activity[1] approximately 200 times greater than the original spoiled hay. These concentrates are essentially free from neutral fats and waxes, certain pigments, sugars, glycosides, water-soluble polysaccharides, water-soluble acids, amines, alkaloids, water-soluble proteins, and the water-soluble decomposition products of chlorophyll. At this stage the concentrates are dark blue-green in color, indicating the presence of pigments arising from the degradation of chlorophyll.[2] The concentrates also contain neutral materials along with *acidic* substances which are water-*insoluble*, but *soluble* in 95 per cent ethanol, methanol, and ether, and which form water-*soluble* sodium salts.

The ratio of physiologically inert materials to the hemorrhagic agent is still too high to permit a practical separation of the latter

[1] Standardized susceptible rabbits were used as the test animals (2, 3).

[2] When sweet clover hays undergo spoilage, the temperature in the stacks rises appreciably. Temperatures between 60–70° have been observed in this work (4). The degradation of the chlorophyll pigments to the porphyrins is apparently quite complete, for in badly spoiled hays the original green color is completely destroyed.

by any of the usual procedures or the more specialized ones like high vacuum distillation, chromatographic adsorption, etc. All attempts to find a chemical test for the hemorrhagic agent which could be correlated with physiological activity as determined by the prothrombin assay were unsuccessful (2, 3, 5).

No useful purpose would be served by listing in detail at this time the numerous and diverse unsuccessful trials which finally led to the fractionation scheme presented below (5). From Step 9 on (see Paper I (1)), the problem of eliminating inert classes of compounds was largely one of freeing the concentrates from certain water-insoluble acidic substances, non-saponifiable substances, compounds with acidic properties containing tertiary nitrogen, acids with basic functional groups, and the higher hydroxy fatty acids soluble in alcohol, ether, and benzene, but insoluble in n-pentane.

The complete removal of the chlorophyll decomposition products without loss of the hemorrhagic agent presented the most formidable problem. After many trials the technique originally employed by Willstätter in his classical researches in this field (6) was found to be the most practical. Willstatter used various concentrations of hydrochloric acid to separate acidic chlorophyll degradation products (phytochlorins and phytorhodins) having tertiary nitrogen groups. Thus these acids are separated from one another by variations *in their basicity* rather than their relative acidity, as is customarily done in the separation of acids. When we applied this technique (to fractionate the crude ether extract from Step 9 (1)) using various concentrations of hydrochloric acid, a series of pigmented fractions was obtained.[2] The pigmented fractions extracted by the acid from the ether solution were for all practical purposes physiologically inert, while the ether solutions, including the one treated with the concentrated (36 per cent) acid (with an olive-yellow color) retained *the bulk* of the original biological activity. These experiments demonstrated unequivocally that the hemorrhagic agent does not have *a basic group*. The presence of an *acidic group* has been indicated previously (see above).   .

After the chlorophyll degradation products were removed via Step 10, and the absence of a basic group in the hemorrhagic agent was established, further fractionation techniques were

concerned largely with the task of removing traces of inert neutral and acidic entrainments. These entrainments, which passed through the extraction process as emulsions, were finally eliminated by extraction with ether, while the hemorrhagic agent was in the form of its water-soluble sodium salt (Step 13). After removal of the non-acidic entrainments the acids were liberated from their sodium salts with hydrochloric acid (Step 14) and shaken out with ether. The residue obtained after the ether was removed possessed an extremely high physiological activity. At this stage the acidic hemorrhagic agent is separated from the higher fatty acids by fractional crystallization from 95 per cent ethanol (Step 15).
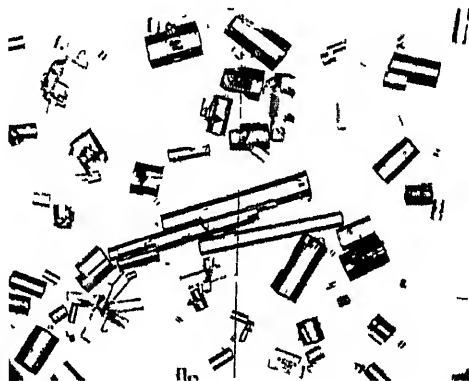
Fig. 1 Hemorrhagic agent from spoiled sweet clover hay (crystallized from cyclohexanone; magnification 67 ✕)

From Step 15 on, the hemorrhagic agent is readily purified by recrystallizing it from a benzene-methanol mixture; after two recrystallizations from acetone the maximum melting point of 288-289° is realized.

In the pure state, the hemorrhagic agent has a low solubility in the common organic solvents, but solution is readily effected in basic media owing to its acidic nature (Step 9 (1)). The pure product (Fig 1) is optically inactive, ash-free, and also free from nitrogen, sulfur, and phosphorus. The molecular diagnosis points to the formula $C_{19}H_{12}O_6$. A crystalline dimethyl ether $C_{19}H_{10}O_4(OCH_3)_2$, m.p. 168-170°, was obtained by methylating with diazomethane.

The substance gives a positive Folin test (7) and forms a colored, coupled product with diazotized sulfanilic acid. The $FeCl_3$ test is negative when made in water or 95 per cent ethanol, but positive in cyclohexanone. Attempts to classify the substance on the basis of its solubility and behavior in the usual characterization schemes failed (8, 9).

The acidic nature of the hemorrhagic agent is reflected in the isolation scheme and its ability to form salts, as well as its behavior in ether towards diazomethane. The acidic properties fall between the usual carboxylic acids and the phenols. If the amount
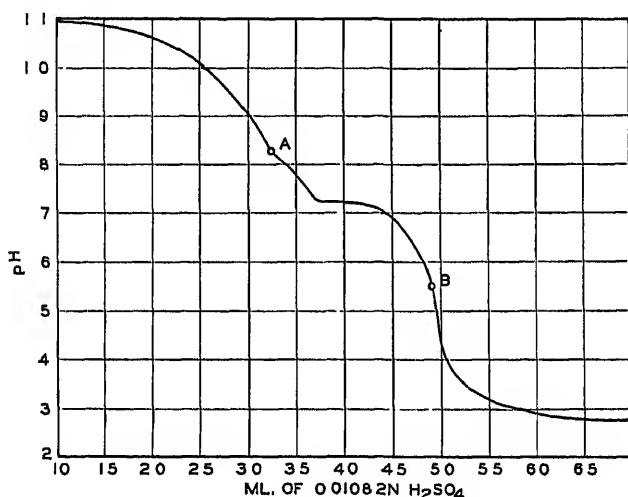
Fig. 2 Electrometric titration curve of the hemorrhagic agent (A and B indicate the inflection points at pH 8 3 and 5 5).

of acid necessary to change the basic solution from pH 8.3 to 5.5 be taken (the points of inflection A and B on the electrometric titration curve, Fig. 2), a neutralization equivalent approximating 160 is obtained. When the substance, dissolved in neutral acetone containing an excess of sodium hydroxide, with phenolphthalein as the indicator, is titrated back, a neutralization equivalent of 168 is obtained.

The physiological activity (prothrombin reduction (2, 3)) of the pure substance is represented by Fig. 3. Experimentally produced hays and supplies of hay encountered in agricultural

practice,[3] which had killed cattle in 1938 and 1939 (5), yield identical products. The yield of the crystalline hemorrhagic agent from the Westfield hay and the experimentally produced hays is essentially the same, and the products have parallel biological activity. These findings are significant, since the prothrombin assay (2, 3) and the entire fractionation scheme were based on work done with spoiled hays produced experimentally in 1934-36 (1, 5).

On the basis of our knowledge of the compound, and of previously reported substances with approximately the same ele-
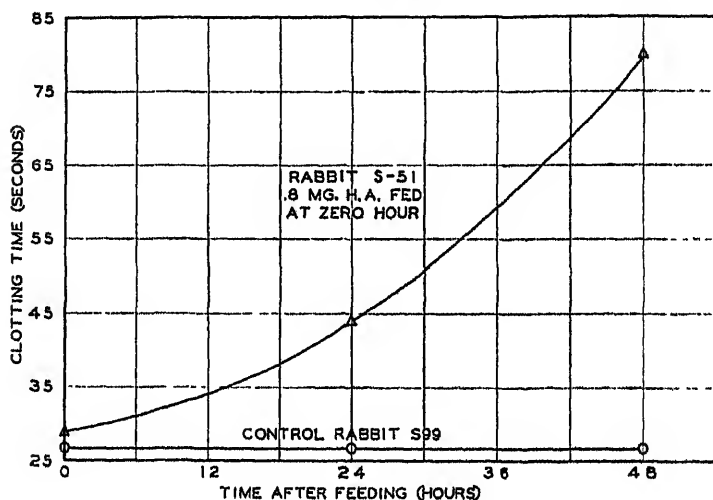
Fig. 3. Increase in the clotting time of the plasma (8 per cent) from Rabbit S-51 after feeding 1.8 mg. of the hemorrhagic agent.

mentary composition, the hemorrhagic agent represents a product which has hitherto *not* been found in nature.[4] The chemical and

[3] Obtained in February, 1939, from a farm near Westfield, Wisconsin. In a herd of twenty cows, seven died and all the others were in a serious hemorrhagic condition when we took charge of the herd and the hay for experimental purposes. These cattle had been eating the hemorrhagic hay *ad libitum* for about a month before the characteristic sweet clover disease symptoms became apparent.

[4] The causal relationship of the total coumarin content of the common strains of sweet clover (*Melilotus alba* and *officinalis*) and the tendency of hay made from them to become hemorrhagic when improperly cured have

physical properties exhibited by the pure substance are as exceptional and anomalous as the disease which it is capable of inducing (12, 13).

In contrast to the known anticoagulants (naturally occurring and synthetic), the hemorrhagic agent from spoiled sweet clover hay appears to have no effect on the clotting powers of normal blood or plasma *in vitro* (14). Its unique physiological activity becomes apparent only when fed by mouth or after injection into the blood stream. Detailed studies on its mode of action and further work on its constitution and structure are in progress.

<div align="center">EXPERIMENTAL</div>

Extraction Procedure (See Diagram)

Step 10—The blue-green ether solution (3 to 4 liters) realized in Step 9 (see Paper I (1)) is shaken in a separatory funnel with four successive 150 ml. portions of concentrated hydrochloric acid (sp. gr. 1.17 (6)). The highly colored acid layers are combined and shaken with fresh ether (150 ml. portions) to remove the entrained hemorrhagic fraction. The ether layers (colored a light yellow or olive-green) are combined. They contain the bulk of the hemorrhagic agent. The dark green hydrochloric acid layers which carry out the phyllo-, pyrro-, and rhodoporphyrins[2] and the solids which separate from the ether layer usually contain entrainments of the hemorrhagic agent, but it is not practical to attempt to remove the small quantity lost.

Step 11—The ether solution from Step 10, which is now essentially free from pigments of the chlorophyll class, is concentrated to dryness. About three-fourths of the ether can be removed by distillation at atmospheric pressure; the balance is removed under reduced pressure at 25–28° in an atmosphere of nitrogen or carbon dioxide. The residue is taken up in 10 ml. of benzene, in which the bulk of the hemorrhagic agent dissolves. Some physiological activity is lost in a dark gummy residue which is insoluble in the benzene.

---

already been pointed out in previous publications by us (10). However, it can be stated with certainty that the hemorrhagic agent described in this communication is not identical with any of the 60 or more coumarin derivatives previously isolated from various species of plants including the different members of the *Melilotus* family (11).

Step 12—The benzene solution from Step 11 is diluted with 25 volumes of Skellysolve A. A brown solid separates which is removed by filtering through a layer of Filter-Cel on a Buchner funnel. The Skellysolve A solution is distilled under reduced pressure; the residue is taken up in benzene, and Skellysolve A is added again.

Steps in Concentration of Hemorrhagic Agent

Ether solution (from Step 9 (1))

|
10. Extract 4 times with HCl (36%)
|

HCl extract (discard)　　　　　11. Ether solution concentrated to dryness; residue taken up in benzene

Insoluble material (discard)　　12. Add Skellysolve A to benzene solution; concentrate filtrate to dryness; dissolve in benzene, again add Skellysolve A

Ppt. (discard)　　　　　　　　13 Concentrate filtrate to dryness; take up in 0.5% NaOH, extract with ether

Ether solution (discard)　　　　14. Acidify alkaline aqueous solution, extract with ether

Aqueous solution (discard)　　　15. Concentrate ether solution, add 95% ethanol; dense crystals are collected by filtration; m.p. 250-255°

Ethanol filtrate, stearic, oleic acids, etc. (discard)　　　16. Crystals are recrystallized from benzene-methanol mixture; m.p. 270-280°; recrystallized from acetone (2 times), m.p. 288-289° (maximum physiological activity)

Step 13 The Skellysolve A solution obtained from Step 12 is finally concentrated to dryness under reduced pressure at 25-28°, yielding a residue that contains appreciable quantities of non-acidic substances. These are removed by shaking out a solution of the sodium salts with ethyl ether. The residue is taken up in about 200 ml. of 0.5 per cent sodium hydroxide. It is then shaken carefully with several portions of ethyl ether. If a stable emulsion is formed, the solution is again made acid with dilute hydrochloric acid. By adding the sodium hydroxide to the acidic

solution carefully the troublesome emulsions are avoided. The hemorrhagic fraction is in the aqueous solution that contains the sodium salts. The ethereal solution of the *non-acidic material* carries no physiological activity.

Step 14—The alkaline solution from Step 13 is acidified with dilute hydrochloric acid. The liberated acidic material forms a cloudy suspension. This is separated from the aqueous solution by shaking the suspension with ethyl ether in a separatory funnel. The aqueous layer is discarded.

Step 15—For crystallization of the hemorrhagic agent the ether solution from Step 14 is concentrated to a volume of about 1 ml., to which 5 ml. of 95 per cent ethanol are added. At this stage dense crystals form spontaneously at the bottom of the vessel. They are collected by filtration and are thus separated from the fatty acids (physiologically inactive) which also crystallize at this point when the solution is cooled to room temperatures.

Step 16—The crude greenish yellow crystals (6.8 mg.) which melt over the range 250–255° are *physiologically active* (reduce the prothrombin level). By recrystallizing from a benzene-methanol mixture, the melting point is raised to the 270–280° range. After two recrystallizations from acetone the melting point remains constant at 288–289°.

After the first crystalline hemorrhagic material was obtained, as indicated above, subsequent operations yielded the same crystals in Step 14 at the interface between the water and the ether. Later it was found that the hemorrhagic agent could also be removed from the insoluble fractions of Steps 11 and 12 with the methanol-ether mixture. On seeding this solution crystallization occurred promptly.

Yields of Hemorrhagic Agent—6 mg. of the pure substance were realized from 3.0 kilos of the hemorrhagic hay in the first successful extraction. The next three separations yielded 16, 18, and 23 mg. respectively. Finally the yields rose to 60 mg. in those instances when all of the sixteen operations demanded by the fractionation scheme were executed successfully.

Analysis and Properties of Hemorrhagic Agent

Melting Point—Capillary, 288–289°.

Optical Rotation—$[\alpha]_D^{25} = 0°$ in benzene; $C = 0.17$ in a 4 dm. tube.

Qualitative Analysis—Ignition test, negative, ash-free; Beil-stein's test, negative, no halide; fusion with KNO_3-Na_2CO_3, negative, no phosphorus; Emich's nitrogen test, negative, no nitrogen.

Quantitative Analysis—13.940 mg. lost approximately 8 γ; the volatile matter was less than 0.1 per cent (Abderhalden drier, 1 mm. of Hg, 78°, 10 hours).

Carbon and Hydrogen Determinations—We are indebted to Dr. H. K. Alber, Biochemical Research Foundation, Philadelphia, for the determinations listed under Analyst B as well as for control qualitative tests which confirmed all of our findings. On a dried sample basis, the results, measured in per cent, were as follows:

	Analyst A		Analyst B	
Carbon found	67.70	67.90	67.84	67.76
Hydrogen found.	3.50	3.60	3.49	3.45
Oxygen (by difference)	28.80	28.50	28.67	28.79

The ultimate analysis and the molecular weight figures (see below) suggest that the empirical formula is in the range $C_{19}H_{12}O_6$.

Molecular Weight by Micro-Rast Method—The substance shows no appreciable solubility in camphor. Its solubility in borneol is sufficiently high so that this medium can be used. The average value of 322 was obtained from several determinations. This value is undoubtedly low, since the hemorrhagic agent undergoes some decomposition in the borneol melt.

Neutralization Equivalent—An excess of 0.148 N NaOH was added to the hemorrhagic agent dissolved in neutral acetone. After the solution had stood for 12 hours it was titrated back with standardized HCl with phenolphthalein as the indicator (pH 9.0); 6.20 mg. required 2.47 ml. of 0.148 N NaOH. This indicates a neutralization equivalent of 168.

Solubility—The pure crystals, m.p. 288–289°, are slightly soluble in benzene (10 mg. per ml. at 76°). Their solubility in acetone and ethyl ether is somewhat lower. In cyclohexanone approximately 8 mg. dissolve in 1 ml. at 25°. The substance shows no substantial solubility in the other common alcohols, ethers, ketones, glycols, and hydrocarbons. Solution is readily effected in pyridine (salt formation) and also in quinoline. The hemorrhagic agent partitions between ethyl ether and dilute bicarbonate solution. Dilute Na_2CO_3 or NaOH dissolves about

1 mg. per ml. In alkaline solution above 12 per cent concentration, the sodium salt is precipitated (salted-out).

Tests for Functional Groups—A positive reaction is obtained with the Folin phenol reagent (7). The ferric chloride test is negative when made in water or 95 per cent ethanol, but positive in cyclohexanone. No reducing action on warm alkaline solutions of silver and cupric salts is observable. On coupling with diazotized sulfanilic acid, a colored product is obtained. All other qualitative tests gave negative results (8, 9).

Electrometric Titration of Hemorrhagic Agent (Fig. 2)—30 mg. were dissolved in 5 ml. of 0.1065 N NaOH. The acidic parent substance was then liberated by titrating with 0.0108 N H_2SO_4. The titration curve was followed by pH measurements with a glass electrode.

Crystal Habit—When analytically pure, the hemorrhagic agent crystallizes from benzene, acetone, and cyclohexanone as hexagonal prisms (see Fig. 1).

Preparation of Dimethyl Ether—The hemorrhagic agent can be methylated with diazomethane and yields a non-acidic dimethyl ether. Attempts to methylate it with dimethyl sulfate failed.

To 40 ml. of dry ether, 34.0 mg. of the hemorrhagic agent were added, whereupon the resulting suspension was cooled to $-4°$. Diazomethane (1.4 gm. in ethyl ether) was distilled into the chilled solution. The ether suspension which contained diazomethane in excess was allowed to stand at 25° for 36 hours.

The dimethyl ether separated as a finely divided floc. After the excess diazomethane was removed under reduced pressure, the ether suspension was treated with 0.5 per cent NaOH to remove the acidic unchanged starting material. The non-acidic fraction (the methylated product) was collected on a filter and washed thoroughly with ether. As obtained the methylated product is analytically pure. By recrystallization from a large volume of methanol, crystals are obtained, which melt at 168–170°. The dimethyl ether does not show the color tests exhibited by the parent substance.

Analysis—Carbon found, 69.18, 69.16; hydrogen found, 4.40, 4.37; equivalent to $C_{21}H_{16}O_6$, mol. wt. 364. Mol. wt. (micro-Rast) with borneol, 340 (average). Methoxyl content, calculated for $C_{19}H_{10}O_4(OCH_3)_2$, 17.03; found, 17.15, 17.20.

Physiological Activity (Prothrombin Reduction)

Bioassay of Crystalline Hemorrhagic Agent (2, 3)—1.8 mg. fed to Rabbit S-51 (with a relative clotting index of 0.50 at the time of standardization[5]) gave an index of 0.50 after 24 hours and a relative clotting index of 0.10 after 40 hours.

Control dosage experiments made with rabbits whose relative clotting index values are between 0.50 and 0.30 at the time of standardization indicate that the reduction in the prothrombin level or activity realized with 50 gm. of the hemorrhagic hay sample (one dose, Paper II) is approximately equivalent to the reduction caused by 1.5 mg. of the pure crystals.

The increase in the clotting time effected by 1.8 mg. with Rabbit S-51 is indicated in Fig. 3. This graph is representative of the response realized when rabbits with approximately the same sensitivity (relative clotting index = 0.50 at the time of standardization) are fed 60 gm. of the hemorrhagic hay sample instead of 50 gm. as in the standardization procedure (2).

Bioassay of Dimethyl Ether—5 mg. were fed to standardized susceptible rabbits with a relative clotting index of 0.50 to 0.30 at the time of standardization. No reduction in prothrombin level or activity resulted from this dosage. When the dimethyl ether was treated with alcoholic KOH at 25°, an acidic fraction was obtained which exhibited some physiological activity. This treatment most likely resulted in a partial demethylation of the ether. Since the ether does not reduce the prothrombin level, it appears that in the parent substance the free acidic hydroxyl groups are involved in the physiological activity.

Efficiency of Isolation Scheme and Percentage of Hemorrhagic Agent in Spoiled Sweet Clover Hays

With the bioassay data presented above as the basis for the calculation, the percentage of the hemorrhagic agent in the spoiled hays used during the past 6 years can be expressed. The experimentally produced spoiled hays from *Melilotus alba* with the highest physiological activity, as well as the Westfield, Wisconsin,[3] hay which killed cattle, contain approximately 0.003 per cent of the hemorrhagic agent on the dry substance basis. Since

---

[5] The relative clotting index is the ratio of the concentration of the normal plasma in the concentration range of 12.5 to 8.34 per cent to the concentration of the pathic plasma which gives the same clotting time (2).

the prothrombin-reducing action of 50 gm. of the hay is about the same as 1.5 mg. of the pure hemorrhagic agent, 3 kilos of the spoiled hays (the quantity employed in each extraction) contain approximately 90 mg. With the highest yields of the pure product realized consistently as the basis for the calculation (60 mg. per extraction), the over-all recovery is 66 per cent. This yield is from many standpoints extraordinary when all the contingencies are considered.

SUMMARY

1. The hemorrhagic agent in spoiled sweet clover hay (*Melilotus alba*) has been isolated in a pure state, m.p. 288–289°, from experimentally produced spoiled hays as well as from hays that killed cattle in agricultural practice.

2. The substance has the empirical formula $C_{19}H_{12}O_6$. It is optically inactive. There are two acidic hydroxyls in the molecule. The acidity of the pure substance falls between that of the phenols and the carboxylic acids. A crystalline dimethyl ether $C_{19}H_{10}O_4(OCH_3)_2$ with a melting point of 168–170° (physiologically inactive) has been prepared by methylation with diazomethane.

3. In the pure state the substance has a low solubility in the ordinary organic solvents. It is insoluble in acid media. Basic solvents and dilute alkali effect solution readily (salt formation).

4. The substance could not be characterized or identified on the basis of its behavior towards the usual identification reagents. Its occurrence in nature has not previously been reported.

5. 1.5 mg. of the crystalline hemorrhagic agent cause approximately the same reduction in the prothrombin level or activity of the plasma of standardized susceptible rabbits in 40 hours as 50 gm. of the standard spoiled hay sample.

6. Spoiled sweet clover hays produced experimentally from *Melilotus alba* and those realized in agricultural practice contain approximately 0.003 per cent of the hemorrhagic agent on the dry substance basis. The over-all yield of the substance in a fractionation scheme involving sixteen steps approximates 66 per cent of the quantity present.

During the 6 years that have elapsed since the first experiments on the isolation of the hemorrhagic agent were begun, various

members of my laboratory not included as authors have contributed much to this work in a very willing manner. Our thanks are due to Dr. E. W. Schoeffel for conducting the initial extraction tests (1934) involving organic solvents. Mr. Lothar Joos assisted in the preparation of crude concentrates from July, 1938, to April, 1939. Mr. Ralph Overman has helped with the bioassays since July, 1938. The molecular weight determinations of the hemorrhagic agent and the photomicrograph were made by Mr. Mark Stahmann. Mr. William Sullivan made the electrometric titration curves. Mr. C. F. Huebner and Mr. Mark Stahmann repeated the entire isolation scheme independently after the first crystalline material was obtained and the former also developed the technique for the preparation of the crystalline dimethyl ether of the hemorrhagic agent. Finally, Mr. R. J. Dimler and Mr. Mark Stahmann assisted in the preparation of the manuscripts for publication. (K. P. L.)

BIBLIOGRAPHY

1. Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *J. Biol. Chem.*, **136**, 47 (1940).
2. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941).
3. Campbell, H. A., Smith, W. K., Overman, R. S., and Link, K. P., *J. Agric. Research*, in press (1941).
4. Roberts, W. L., Summaries of doctoral dissertations, 1936–37, Madison, **2**, 204–206 (1938).
5. Campbell, H. A., Summaries of doctoral dissertations, 1939–40, Madison, **5**, 155 (1940).
6. Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin (1913).
7. Folin, O., and Denis, W., *J. Biol. Chem.*, **12**, 239 (1912).
8. Staudinger, H., Anleitung zur organischen qualitativen Analyse, Berlin, 2nd edition (1929).
9. Meyer, H., Analyse und Konstitutionsermittlung organischer Verbindungen, Berlin, 5th edition (1931).
10. Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **119**, 269 (1937); *Ind. and Eng. Chem., Anal. Ed.*, **9**, 438 (1937).
11. Spáth, E., *Ber. chem. Ges.*, **70**, 83 (1937); *Monatsh. Chem.*, **69**, 75 (1936).
12. Roderick, L. M., and Schalk, A. F., *North Dakota Agric. Exp. Stat., Bull. 250* (1931).
13. Roderick, L. M., *Am. J. Physiol.*, **96**, 413 (1931).
14. Luck, J. M., and Hall, V. E., Annual review of physiology, Stanford University, **1**, 297 (1939).

·

THE FORMATION OF ACETYLMETHYLCARBINOL FROM PYRUVIC ACID BY A BACTERIAL ENZYME PREPARATION

By M. SILVERMAN AND C. H. WERKMAN

(*From the Bacteriology Section, Industrial Science Research Institute, Iowa State College, Ames*)

The present contribution reports the preparation of an active cell-free bacterial juice capable of converting pyruvic acid into carbon dioxide and acetylmethylcarbinol. Aside from the importance to be attributed to the preparation of an active juice from bacteria for studies of intermediary metabolism, the action of the juice is of theoretical interest in that it represents a biological synthesis of a 4-carbon from a 3-carbon compound; *i.e.*, an assimilative phase brought about by a cell-free bacterial enzyme preparation. The system represents an isolated pyruvate mechanism and differs from any previously reported.

Neuberg and Kobel (1) have maintained that two enzyme systems, carboxylase and carboligase, are involved (Equations 1 and 2).

$$2CH_3COCOOH \xrightarrow{\text{carboxylase}} 2CO_2 + 2CH_3CHO \qquad (1)$$

$$2CH_3CHO \xrightarrow{\text{carboligase}} CH_3COCHOHCH_3 \qquad (2)$$
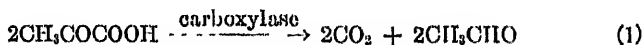
The existence of carboligase has been questioned by other workers (Dirscherl (2); Tanko and Munk (3)). We shall compare the properties reported in the literature for carboxylase with those of the acetylmethylcarbinol system extracted from *Aerobacter aerogenes*.

Methods

Pyruvic acid was determined manometrically by ceric sulfate oxidation. At the conclusion of the experiment an aliquot of the

fermentation liquor (containing less than 2.0 mg.) was acidified with sulfuric acid to remove any carbon dioxide. The sample was placed in the main vessel of Warburg cups and 0.5 ml. of saturated ceric sulfate solution in N sulfuric acid added to the side arm. After temperature equilibrium was reached, the contents were mixed and the reaction was permitted to continue for 2 hours with constant shaking at 30°, when the carbon dioxide evolved was calculated. 1 mM of pyruvic acid corresponds to 22,400 c.mm. of carbon dioxide. No lactic acid was formed by the enzyme. Known quantities of pyruvic acid gave recoveries of 98 to 102 per cent.

Acetylmethylcarbinol was determined by the method of Stahly and Werkman (4). Carbon dioxide was determined manometrically, residual carbon dioxide being released by acidification with sulfuric acid.

The manometric methods of Barcroft and Warburg were used, modified according to Dixon (5). In the macromanometric experiments the type of vessel described by Wood and Werkman (6) was employed. The experiments were conducted at 30.4° unless otherwise specified.

All chemicals employed were reagent grade. Synthetic thiamine and cocarboxylase were kindly supplied by the Winthrop Chemical Company, Inc., and Merck and Company, Inc., respectively. Pyruvic acid was employed as the sodium salt.

Acetaldehyde was determined by the bisulfite titration of Donnally (7). The acetaldehyde was separated from pyruvic acid by refluxing and aeration into bisulfite. The bisulfite fixed was determined by iodine titration.

Enzyme Preparation

Aerobacter aerogenes (Strain 174) was grown in 5 liter quantities of nutrient broth made up with distilled water and containing 1.0 per cent glucose, 0.3 per cent proteose-peptone, 0.8 per cent dipotassium phosphate, and 10 per cent tap water. The phosphate and tap water were sterilized separately. The medium was inoculated with the growth from a single agar slant and incubated 20 hours at 30°. The cells were recovered by centrifugation in a continuous Sharples supercentrifuge at about 35,000 R.P.M. The yield from 5 liters of broth varied between 15 and 20 gm. of

cell paste which was employed without further washing. The procedure is based on that described by Wiggert *et al.* (8), except that either M/15 potassium acid phosphate or distilled water was employed in grinding and extraction. The bacteria are disrupted by grinding with powdered glass in the proper proportions and subsequent extraction of the ground mass with phosphate buffer or water. From 15 gm. of cell paste about 30 ml. of active juice may be obtained by three successive extractions with 10 to 12 ml. of buffer or water.

Table I shows the relative activity of the first three successive extractions of the ground glass-cell paste mixture as indicated by the rate of carbon dioxide evolution from pyruvic acid.

The juices were frozen and retained a nearly undiminished

TABLE I

Relative Activity of Successive Extractions of Enzyme Preparation

Substrate, 6 mg. of pyruvic acid, atmosphere, N_2; enzyme preparation, 0.15 ml.; buffer, 1 ml. of M/15 KH_2PO_4; volume, 2.00 ml.

Interval	CO_2 evolution		
	Extract 1	Extract 2	Extract 3
min.	*c.mm.*	*c.mm.*	*c.mm.*
0–15	552	310	73
15–30	216	226	122

activity for 2 months; after 6 months their activity was still appreciable.

Products of Pyruvic Acid Dissimilation

The products of the breakdown of pyruvic acid were carbon dioxide and acetylmethylcarbinol. No hydrogen, acetic acid, ethyl alcohol, or 2,3-butylene glycol was detected. The ratio of pyruvic acid utilized to carbon dioxide and acetylmethylcarbinol formed is approximately 2:2:1 (Table II). The general equation is

$$2CH_3COCOOH \longrightarrow 2CO_2 + CH_3CHOHCOCH_3 \qquad (3)$$
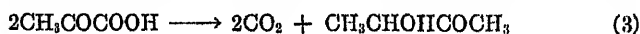
In a typical fermentation of pyruvic acid by cells of *Aerobacter*, 2,3-butylene glycol, acetic acid, hydrogen, and occasionally formic acid occur in addition to carbon dioxide and the carbinol. Apparently the hydroclastic enzyme catalyzing the reaction

$CH_3COCOOH + HOH \rightarrow CH_3COOH + HCOOH$ is inactivated in the enzyme preparation. The removal of the hydroclastic system greatly simplifies the study of the formation of acetylmethylcarbinol from pyruvate.

TABLE II

Dissimilation of Pyruvate by Juice of Aerobacter aerogenes

Juice, 3 ml. of Extract 1 (fresh juice); buffer, 17 ml. of M/15 KH$_2$PO$_4$; volume, 30 ml.; duration of experiment, 3 hours.

Pyruvic acid			CO$_2$	Acetylmethyl-carbinol
Initial	Final	Utilized		
m_M	m_M	m_M	m_M	m_M
2.49	0.56	1.93	1.80	1.00
9.17	1.60	7 57	7 63	3.30
4.99	1.08	3.91	3 62	1.61
2 49	0.88	1.61	1.40	0 83
5.00	0.50	4.50		2.33
9.17	1.69	7.48	7.42	3.40

TABLE III

Effect of Temperature on Rate of Reaction

Substrate, 45 mg. of pyruvic acid; atmosphere, air (manometric investigations showed the enzyme could not utilize O_2, an atmosphere of air was employed in all work following); juice, 0.15 ml. (two separate samples were employed); buffer, 0.3 ml. of M/15 KH$_2$PO$_4$; volume, 2.0 ml.

Enzyme preparation No.	CO$_2$ evolved in 15 min.											
	18 4°	20.4°	22.4°	24.4°	26.4°	28.4°	30.4°	32 4°	34.4°	36.4°	38.4°	40.4°
	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c mm.	c.mm.	c.mm.	c.mm.	c mm.	c.mm	c.mm.
1	151	176	256	343	366	415	464	467	497	530		
2						480	497	521	556	540	512	490

Physical Properties of Enzyme Preparation and Determination of Optimal Conditions

Temperature Relations—Although 30° is reported as the optimal growth temperature for *Aerobacter*, that for the activity of the enzyme preparation is somewhat higher. Under the conditions of Table III the temperature optimum ranges between 34–36°. Neuberg (9) reports that yeast carboxylase is active over the range

of 8° to 65°. The preparation when heated for 5 minutes at 65° retained only 6 to 7 per cent of its activity. Melnick and Stern (10) report that 5 minutes at 60° reduced the activity of yeast carboxylase 96 per cent.

Effect of pH on Enzyme Activity—Mickelson and Werkman (11) have previously reported that in the fermentation of glucose by *Aerobacter* acetylmethylcarbinol and 2,3-butylene glycol were formed only at acid reactions. Our enzyme preparation yielded

TABLE IV

Effect of pH on Rate of CO_2 Formation

Substrate, 188 mg. of pyruvic acid; enzyme preparation, 0.15 ml.; buffer, 1 ml. of M/15 PO_4; volume, 2 ml.

Experiment No.		CO₂ evolved								
		pH 4.5	pH 5.6	pH 6.0	pH 6.4	pH 6.8	pH 7.2	pH 7.6	pH 8.0	pH 6.4*
		c. mm.	c. mm.	c. mm.	c. mm.	c. mm.	c. mm.	c. mm.	c. mm.	c. mm.
1	0–15 min.	248	234	240	183	72	12	6	7	4
	15–30 "	66	75	55	64	65	18	3	2	0
	0–169 "	413	418	398	375	299	173	64	58	12
	CO₂ from ceric oxidation	42	52	51	80	152	277	401	398	
	Sum	455	470	449	455	451	450	465	456	
	Theoretical	478								
	Voges-Proskauer test	4+	4+	4+	4+	4+	4+	?	?	—
2	0–15 min.	170	122	119	76	25	11	4	9	
	Voges-Proskauer test	4+	4+	4+	4+	2+	?	—	—	
3	0–15 min.	47	107	101	81	25	7	9	3	3
	Voges-Proskauer test	4+	4+	4+	4+	4+	+	—	—	—
4	0–15 min.	108	120	122						
5	0–15 "	164	221	234						

* Endogenous.

similar results (Table IV). Above pH 7.2 little or no carbon dioxide is evolved and no carbinol is formed. The Voges-Proskauer test is negative (creatine and 40 per cent potassium hydroxide). It is also evident that at alkaline levels the pyruvate was not metabolized by any other mechanism, since the pyruvate can be recovered unchanged (Table IV, Experiment 1, fourth line). The optimal pH range appears to be 5.6 to 6.0. Below pH 5.6 the results were irregular, some preparations being equally active at pH 4.5 and 5.6, whereas others were less active at pH 4.5 (Experi-

ments 3 and 5).   Melnick and Stern (10) found that pH 4.8 inhibits carboxylase activity in yeast about 80 per cent.   This decrease in activity is much greater than was observed with our preparation at similar pH levels.

Mickelson (12) has shown that pyruvate undergoes hydroclastic cleavage by *Aerobacter* at alkaline pH levels, yielding acetic and formic acids in equimolar proportions.   The dissimilation appears to be the result of competition between the acetylmethylcarbinol and the hydroclastic systems for the pyruvic acid wherein the pH determines the path of dissimilation.   This may be shown manometrically with a cell suspension, since both hydrogen and carbon dioxide arise from the formate produced in the hydroclastic reaction, but only carbon dioxide occurs as the gaseous product in the enzymatic formation of acetylmethylcarbinol.   As the system

TABLE V

pH and Gas Ratios of Aerobacter aerogenes (Whole Cells) on Pyruvate

Substrate, 2.2 mg. of pyruvic acid; atmosphere, N_2; cells, 24 hours old, approximately 1 per cent; buffer, M/15 PO_4, 1 ml.; volume, 2.3 ml.

	pH 4 5	pH 5.6	pH 6.8	pH 8.0
	c mm.	c mm.	c.mm.	c.mm.
H_2................ .	164	208	335	370
CO_2.	426	449	465	398
CO_2:H_2..	2.60	2 16	1.39	1.08

becomes more alkaline, more hydrogen should be evolved, since the acetylmethylcarbinol system is inhibited, whereas the hydroclastic cleavage still operates.   The ratio of carbon dioxide to hydrogen will be 1 when the hydroclastic enzyme only is functioning (Table V).   At pH 8.0, the acetylmethylcarbinol system is completely inhibited.

The experiments of Table V were carried out in double side arm Warburg vessels.   One side arm contained 0.3 ml. of 2 N carbon dioxide-free sodium hydroxide in which the evolved carbon dioxide was absorbed.   After the hydrogen values were obtained, 0.3 ml. of 10 N sulfuric acid was introduced into the side arm which had contained the pyruvate and after temperature equilibrium was reached, the acid was mixed with the contents of the main chamber and sodium hydroxide in the side cup to liberate the carbon

dioxide formed by the fermentation. It is essential that both the hydrogen and carbon dioxide be determined in the same vessel, for the breakdown of formic acid is a reversible reaction.

Effect of pH on Formation of Enzyme—Acidity of the growth medium is essential for the formation of the carbinol enzyme system in the bacterial cell. When the organism is grown between pH 7.5 and 8.5, little or no enzyme develops. In Fig. 1 are shown the relative activities of enzyme preparations made from "alkaline-grown" and from "acid-grown" cells. The "acid" cells were grown as usual; the "alkaline" cells on the same medium plus additions of potassium diphosphate and N sodium hydroxide to
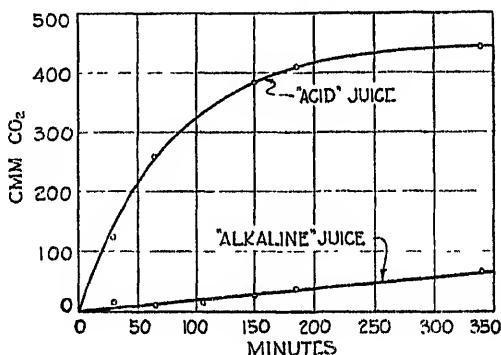
FIG. 1. Comparison of juices from acid- and alkaline-grown cells. Enzyme preparation, 0.1 ml.; buffer, 1.0 ml. of M/15 PO_4, pH 5.6; volume, 2.0 ml., substrate, 2.64 mg. of pyruvic acid.

maintain the pH at 7.5 or higher. The two preparations differed only in the pH of the medium in which the cells were grown.

It is apparent from Fig. 1 that either much more of the carbinol enzyme or a much more active enzyme is present in the juice from "acid" cells. The change in the pH of growth does not cause a change in the pH optimum of the carbinol enzyme. The pH of growth affects primarily the quantity or activity of the enzyme produced.

It was of interest to see whether the production of the carbinol system could be completely suppressed by continuous serial transfer of the organism in alkaline media. If such were the case, *Aerobacter* would be converted into essentially *Escherichia coli*.

Aerobacter aerogenes was carried through 75 transfers by loop inoculation in a medium of 0.75 gm. of proteose-peptone, 0.3 gm. of glucose, c.p., 5.0 gm. of potassium diphosphate, 25 ml. of tap water, and 225 ml. of distilled water. No acetylmethylcarbinol was formed; yet after the 75th transfer when the organism was grown on Difco Voges-Proskauer medium at an acid pH acetylmethylcarbinol was formed. This would indicate that the ability to form the enzyme is retained and that its production is conditioned by the pH of the medium.

Optical Rotation of Acetylmethylcarbinol—A sample of fermentation liquor containing 0.043 gm. of carbinol in 5 ml. was clarified by precipitation of the protein with sulfuric acid. With a 2 dm. tube the rotation of acetylmethylcarbinol was $\alpha_{5460} = -1.72°$; therefore the specific rotation

$$[\alpha]_{5400} = \frac{\alpha \times \text{volume (ml.)}}{1 \text{ (dm.)} \times \text{weight (gm)}} = \frac{-1.72° \times 5}{2 \times 0.043} = -100.0°$$

The rotation of an unincubated mixture of enzyme and pyruvic acids was negligible. Lowry and Baldwin (13) report the rotation of liquid acetylmethylcarbinol as $[\alpha]_{5401} = -6.6°$. By fractional distillation $[\alpha]_{5461}$ could be increased to $-8.0°$. The differences between our value and those of Lowry and Baldwin may be due to different concentrations of inactive polymeride in the samples employed. For example, Dirscherl and Schöllig (14) have reported values for $[\alpha]_D^{20}$ varying from $-33.7°$ to $-103°$.

Effect of Centrifugation and Filtration—The carbinol enzyme is completely retained by Jena glass, Seitz, or Chamberland filters. On the other hand, high speed centrifugation does not decrease its activity. After 1 hour's centrifugation at 250,000 to 300,000 r.p.m. in a simple Beams air-driven ultracentrifuge, the rotor of which is 1.25 inches in diameter, the activity of the supernatant is not appreciably reduced. Not only is the rate of carbon dioxide production maintained, but the ability to produce acetylmethylcarbinol is not diminished.

Effect of Inhibitors

If, as Neuberg maintains, two enzymes, carboxylase and carboligase, are involved in the enzymatic production of acetylmethylcarbinol, it should be possible to inhibit the carboligase

selectively without affecting the activity of carboxylase. In such a case we should obtain CO_2 and acetaldehyde but no acetylmethylcarbinol from pyruvic acid. All inhibitors of carbinol formation which we have used, however, also inhibited CO_2 production (Table VI). Destroying the carbinol enzyme by heat also stops CO_2 production. We found no evidence for the existence of separate enzymes. The degree of inhibition varies somewhat with each enzyme preparation.

TABLE VI

Effect of Various Inhibitors

Substrate, 6 mg. of pyruvic acid; atmosphere, N_2; enzyme preparation, 0.15 ml.; buffer, 1 ml. of $M/15$ KH_2PO_4; volume, 2.00 ml.

Inhibitor	Final molarity	Activity in 1 hr.	Per cent inhibition of CO_2 production	Voges-Proskauer test
		c.mm. CO_2		
None		353		4+
Sodium fluoride	0.005	237	36	3+
Ethyl carbamate.	0.05	109	61	−?
Potassium cyanide.	0.05	204	45	2+
	0.01	120	68	?
α-Bromopropionic acid	0.125	225	39	2+
Sodium arsenite	0.033	58	83	−
" azide	0.044	42	88	−
	0.022	117	67	?
Iodoacetate .	0.007	53	85	−

Acetaldehyde As Intermediate in Formation of Acetylmethylcarbinol

If, according to Neuberg, carboligase participates in the formation of acetylmethylcarbinol, we should expect the enzyme to utilize acetaldehyde. This is not the case. Acetaldehyde added to fermentation of pyruvic acid did not increase the carbinol yield but was quantitatively recovered (Table VII). Slight decreases in acetaldehyde are experimental.

That acetaldehyde is not an intermediate is strengthened by the fact that Mickelson (12) was unable to fix any acetaldehyde by means of sulfite, bisulfite, or dimedon in dissimilations of pyruvate by *Aerobacter*. No attempt was made to fix acetaldehyde in pyruvate fermentations by the enzyme preparations, since the

fixatives named are very toxic. From the data in Table VII it may also be concluded that no acetaldehyde is formed by decarboxylation, since in no case is there any increase over the initial acetaldehyde concentration.

<center>TABLE VII</center>

<center>*Effect of Acetaldehyde on Acetylmethylcarbinol Formation*</center>

Juice, 3 ml. of Extract 1 (fresh juice); buffer, 17 ml. of M/15 KH₂PO₄; volume, 30 ml.; duration of experiment, 12 hours; temperature, 18.5°.

Experiment No.	Pyruvic acid utilized	Acetaldehyde		Acetylmethyl-carbinol
		Initial	Final	
	mM	mM	mM	mM
21	2.32			0.97
22	2.33			1.09
23	2.31	0.89	0.86	1.10
24	2.30	1.78	1.72	1.04
25	2.28	2.22	2.17	0.96
26	2.31	2.67	2.53	0.93
27	2.27	3.11	3.01	0.94
28	2.28	3.56	3.40	1.07
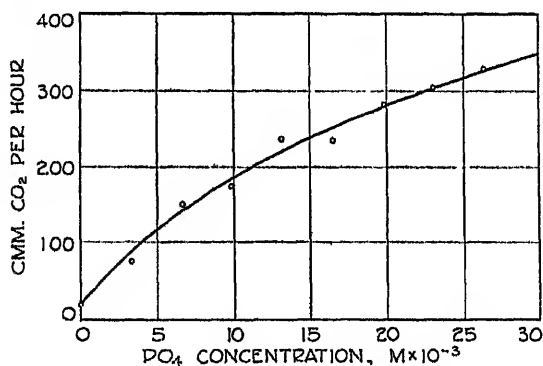
FIG. 2. Effect of PO₄ concentration on rate of pyruvate dissimilation of *Aerobacter aerogenes*. Enzyme preparation, 0.1 ml.; volume, 2.0 ml.; substrate, 8 mg. of pyruvic acid.

<center>*Inorganic Ion Requirements*</center>

The acetylmethylcarbinol enzyme requires inorganic phosphate. Fig. 2 gives the rate of CO_2 production at various phosphate con-

centrations.   In all cases the final buffer concentration was M/30, made up of mixtures of acetate and phosphate buffers.   In Fig. 3 is shown the effect of successive additions of a fixed quantity of phosphate buffered at pH 5.6.   The data indicate that the added phosphate is bound by the system, since the latter is so markedly activated by small additions of phosphate.   We have not been able, however, to demonstrate any direct uptake of phosphate even in the presence of 0.05 M fluoride.   Phosphate determinations were made by the Fiske and Subbarow (15) and Bell and Doisy (16) methods.   Lipmann (17) has indicated that in the breakdown of pyruvate by *Lactobacillus delbruckii* preparations a phosphorylated intermediate occurs and in our case the intermediate may be
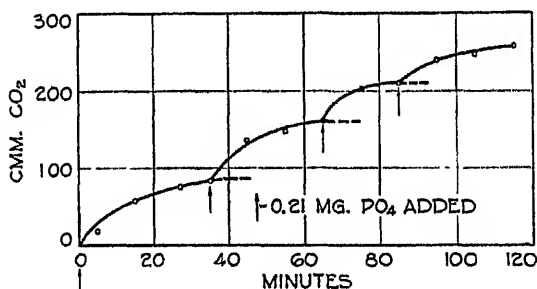
Fig. 3. Effect of PO_4 on pyruvate dissimilation by juice of *Aerobacter aerogenes*.   Enzyme preparation, 0.1 ml.; buffer, 1.0 ml. of acetate, pH 5.6; volume, 1.9 to 2.2 ml.; substrate, 8 mg. of pyruvic acid.

a very labile compound which is hydrolyzed in the course of analysis as suggested by Lipmann.

Acetyl phosphate has been suggested by Lipmann (17) as the possible phosphorylated intermediate in the formation of acetic acid from pyruvic acid.   Employing the carbinol enzyme preparation, we have unsuccessfully attempted to obtain the synthesis of diacetyl- or acetylmethylcarbinol from acetyl phosphate (prepared according to Kammerer and Carius (18)) with formate or acetaldehyde as hydrogen donators.

Magnesium (as the chloride) stimulates the system only slightly, whereas 100 γ of manganese (as the sulfate) increase the rate by about 50 per cent.   The relationship between manganese and cocarboxylase is brought out in Fig. 4.   It is only when cocar-

boxylase is the limiting factor that stimulation by added manganese can be demonstrated. When the system is saturated with cocarboxylase, manganese does not stimulate.

Cocarboxylase

Extract 5 of a ground cell-glass mixture was employed as a source of enzyme. Saturation of the enzyme with cocarboxylase nearly doubles the rate of reaction (Fig. 4). Thiamine is without catalytic effect. Thiamine in the presence of suboptimal amounts
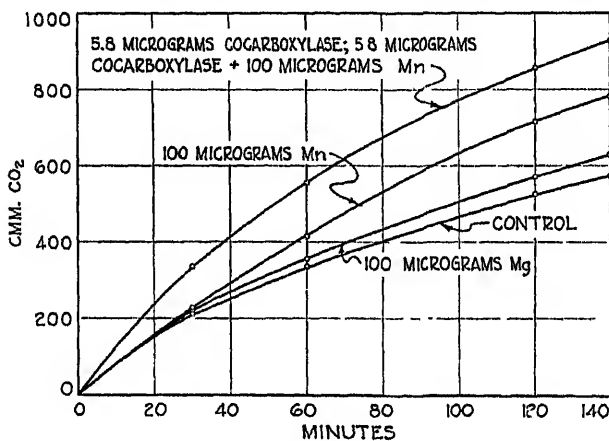
Fig. 4. Effect of cocarboxylase, magnesium, and manganese on activity. Enzyme preparation, 0.1 ml.; buffer, 1.0 ml. of M/15 KH_2PO_4; volume, 2 ml.; substrate, 8 mg. of pyruvic acid.

of cocarboxylase does not stimulate. Westenbrink and van Dorp (19) have shown that thiamine stimulates cocarboxylase activity in alkaline washed yeast by inhibiting phosphatases responsible for the hydrolysis of cocarboxylase. Apparently this enzyme is not present in this preparation.

Free riboflavin and cozymase had no effect. Yeast carboxylase and *Aerobacter* acetylmethylcarbinol systems are alike in their need for cocarboxylase and inorganic ions (20).

DISCUSSION

The carboxylase of yeast and the acetylmethylcarbinol system of *Aerobacter* differ with regard to heat resistance and effect of

pH, but the major difference lies in the need for inorganic phosphate for CO_2 cleavage from pyruvate by the *Aerobacter* system. This taken together with the observation of Lipmann (21) that pyruvate decarboxylation by yeast does not require inorganic phosphate indicates that a carboxylase similar to that of yeast is not a constituent of the *Aerobacter* carbinol enzyme system.

The failure of the system to utilize acetaldehyde indicates the absence of carboligase in the *Aerobacter* preparation. The inability to separate decarboxylation from carbinol production by inhibitor action is further evidence against the existence of an independent carboligase.

The possibility of the existence of two individual enzyme systems, carboxylase and an independent acetylmethylcarbinol system, in the yeast cell should be considered. Much of the confusion regarding the mechanism of formation of acetylmethylcarbinol by yeast may be due to the fact that carboxylase activity is independent of carbinol formation and some phosphorylated intermediate and not acetaldehyde occurs in the formation of acetylmethylcarbinol from pyruvic acid.

SUMMARY

1. A method is described for obtaining an active enzyme preparation from *Aerobacter*, quantitatively catalyzing the conversion of pyruvic acid to carbon dioxide and acetylmethylcarbinol.

2. The preparation acts only at an acid pH and the production of the enzyme in the bacterial cell during growth requires an acid reaction.

3. The hydroclastic and acetylmethylcarbinol systems compete for the available pyruvic acid and the distribution of the latter between the two systems is governed by the pH.

4. The preparation does not use acetaldehyde. From this and the data obtained with inhibitors, it is concluded that carboligase does not participate in the formation of acetylmethylcarbinol by *Aerobacter*.

5. The cleavage of pyruvate requires inorganic phosphate. Since yeast carboxylase does not require inorganic phosphate, the participation of carboxylase is in question.

6. The formation of a phosphorylated intermediate is indicated.

7. Cocarboxylase and manganese (to a less extent magnesium) stimulate the acetylmethylcarbinol system.

8. It is suggested that the enzyme carboxylase may not be involved in the formation of acetylmethylcarbinol by yeast.

BIBLIOGRAPHY

1. Neuberg, C., and Kobel, M., in Oppenheimer, C., Die Fermente und ihre Wirkungen, The Hague, 5th edition, 1329 (1929).
2. Dirscherl, W., *Z. physiol. Chem.*, **252**, 70 (1938).
3. Tanko, B., and Munk, L., *Z. physiol. Chem.*, **262**, 144 (1939).
4. Stahly, G. L., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **10**, 205 (1936).
5. Dixon, M., Manometric methods, London (1934).
6. Wood, H. G., and Werkman, C. H., *Biochem. J.*, **34**, 129 (1940).
7. Donnally, L. H., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 91 (1933).
8. Wiggert, W. P., Silverman, M., Utter, M. F., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **14**, 179 (1940).
9. Neuberg, C., in Oppenheimer, C., Die Fermente und ihre Wirkungen, The Hague, 5th edition, 1327 (1929).
10. Melnick, J. L., and Stern, K. G., *Enzymologia*, **7**, 129 (1940).
11. Mickelson, M., and Werkman, C. H., *J. Bact.*, **36**, 67 (1938).
12. Mickelson, M., Thesis, unpublished, Iowa State College (1939).
13. Lowry, T. M., and Baldwin, W. C. G., *J. Chem. Soc.*, 704 (1935).
14. Dirscherl, W., and Schöllig, A., *Z. physiol. Chem.*, **252**, 53 (1938).
15. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
16. Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, **44**, 55 (1920).
17. Lipmann, F., *Nature*, **144**, 381 (1939).
18. Kammerer, H., and Carius, L., *Ann. Chem.*, **131**, 170 (1894).
19. Westenbrink, H. G. K., and van Dorp, D. A., *Nature*, **145**, 465 (1940).
20. Tauber, H., *J. Biol. Chem.*, **125**, 191 (1938).
21. Lipmann, F., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 248 (1939).

FATE OF THIAMINE IN THE DIGESTIVE SECRETIONS*

By DANIEL MELNICK,† WILLIAM D. ROBINSON,‡ AND HENRY FIELD, JR.

(From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor)

When an oral test dose of thiamine is taken by the normal subject, in the postabsorptive and fasting state, a much smaller fraction of it is excreted in the urine than when the same test dose is ingested immediately after a meal (2). The difference in the urinary thiamine values noted under such circumstances may be due to the instability of thiamine in a more alkaline gastrointestinal tract when gastric secretion is at a minimum. Such a hypothesis is supported by the small urinary thiamine values consistently obtained from patients with peptic ulcer receiving antacid medication and with subjects having achlorhydria (3). The ulcer patients subsisted on diets approaching normal in thiamine content, 700 to 900 γ daily, and furnishing a favorable thiamine to non-fat caloric ratio of approximately 1.0. In unpublished experiments, we have found that the normal individual, subsisting on the 7th day Sippy diet (4), exhibits a gradual decrease in the urinary excretion of thiamine during the 14 days after the hourly addition of antacids (calcium and magnesium carbonates). During the latter part of this period the urinary values are well within the deficiency range. The low values for thiamine in such urine samples have been shown not to be due

to destruction of the vitamin in the bladder prior to excretion because of the alkalinity of the urine (5).

In the present study additional tests were carried out to determine the fate of thiamine *in vitro* after incubation with the digestive secretions[1] under a variety of conditions, similar to those found *in vivo*. In most of the experiments normal digestive secretions were employed. In others, either pathological materials were used or normal samples were modified to simulate the conditions found in pathological organisms. The fate of thiamine in gastric juice in the presence of antacids was also studied. Such an investigation is pertinent to the present study, since the therapeutic value of the antacids depends either upon their alkalinity or adsorbing power. These factors are known to destroy or remove thiamine from solution. Reports by Mahlo (6) and by Schroeder (7), in which thiochrome procedures for the estimation of thiamine were used, state that the vitamin is completely destroyed in gastric juice containing hemin even at an acid pH. Leong (8) had previously found with the rat bradycardia method that added thiamine was stable in blood at pH 7.3 when incubated for 24 hours at 19°, but at 37° a 30 per cent loss did occur. Sinclair (9–11) has repeatedly emphasized that thiamine is unstable in gastric juice in achlorhydria. These problems were also reinvestigated in the present study. For the determination of thiamine the method based upon the reaction between the vitamin and diazotized *p*-aminoacetophenone was used (12). All samples[2] were first subjected to benzyl alcohol extraction as in the case of urine specimens (5).

<center>EXPERIMENTAL</center>

Fate of Thiamine in Gastric Juice—Histamine-stimulated gastric juice[3] from normal fasting subjects was collected in short periods

---

[1] To each 50 cc. sample 1 cc. of toluene was added as a preservative during the period of incubation.

[2] To each 50 cc. sample of gastric and duodenal pancreatic juice, adjusted to pH 4.5, 5 gm. of sodium sulfate were added prior to concentration *in vacuo*. 40 cc. and 20 cc. benzyl alcohol extractions of the residue were carried out. In the case of bile, 10 gm. of sodium sulfate were added to each sample.

[3] Similar results were obtained from gastric juice collected from a normal fasting subject without histamine stimulation.

and any samples containing bile were discarded. The reaction of the gastric juice was adjusted to pH values ranging from 1.5 to 8.5. To each specimen 100 γ of thiamine were added and the solution incubated at 37.5°. The pH values remained practically constant during the periods of incubation. To some of the sam-

TABLE I

Stability and Availability of Thiamine in Gastric Juice with and without Antacids

100 γ of thiamine were added to each specimen except Sample A before incubation.

Sample (50 cc.)	Antacid added	Incubation at 37.5°		Thiamine in solution
		pH*		
			hrs.	γ
A	None	1.5	16	<5
B	"	1.5	16	104
C	"	1.5	40	106
D	"	8.0	16	97
E	"	8.0	40	93
F	"	8 5	16	89
G	"	8.5	40	77
H	Mg$_2$Si$_8$O$_8$†	7.5	6	15
I	"	7.5	16	14
J	"	7.5	40	5
K	CaCO$_3$-MgCO$_3$†	8.5	6	93
L	"	8.5	16	82
M	"	8.5	40	63
N	Al(OH)$_3$†	3.3	16	89
O	"	3.3	40	86

* These initial pH values remained practically constant during the periods of incubation.

† The amounts of the antacids added in each case to the 50 cc. aliquots of the gastric juice sample were 1.0 gm. of the magnesium trisilicate, 2.0 gm. of the carbonates (1:1 ratio), and 10 cc. of a 4 per cent suspension of aluminum hydroxide.

ples antacids[4] were added with no other pH adjustments made. In these tests the suspensions were centrifuged following the

[4] The magnesium trisilicate, calcium carbonate, and magnesium carbonate were obtained in tablet form from Eli Lilly and Company, Indianapolis. These were ground to a powder before addition to the gastric juice. The aluminum hydroxide suspension (amphojel) was obtained from John Wyeth and Brother, Inc., Philadelphia.

various periods of incubation and the antacid precipitates were washed with 50 cc. of distilled water. The thiamine content of each of the pooled solutions and washings was determined.

In Table I the more important analyses are presented. These indicate that thiamine is stable over a period of 16 hours in gastric juice from pH 1.5 to 8.0. Some destruction of the vitamin does occur at a more alkaline pH, but such a reaction is generally be-

TABLE II

Stability of Thiamine in Hemin-Containing Gastric Secretions and in Gastric Secretions in Achlorhydria

Sample	Blood added	pH of incubated material	Thiamine added to 25 cc. aliquot	Thiamine in samples after 16 hrs. incubation at 37.5°	
				Found	Recovery*
	cc.		*γ*	*γ*	*per cent*
Normal	None	4.5	100	104	100
	"	4.5	2500	2370	95
	"	8.0	100	93	89
	"	8.0	2500	2100	84
	0.5†	1.5	100	101	97
	0.5†	1.5	2500	2330	93
	0.5†	4.5	100	97	93
	0.5†	4.5	2500	2400	96
	0.5†	8.0	100	86	83
	0.5†	8.0	2500	2130	85
Achlorhydria	None	7.3	100	99	99
	"	7.5	100	96	96
	"	7.5	2500	2300	92
	"	7.8	100	88	88
	"	7.8	2500	2130	85

* These values were calculated from the figures obtained in recovery experiments with no preliminary incubation of the solutions. Under such circumstances quantitative recovery of the added vitamin is obtained.

† Blood was first diluted 1:3 with water to lake the cells.

yond the physiological range in the stomach even in cases of achlorhydria. In the presence of the magnesium trisilicate there is almost complete loss of the vitamin due entirely to adsorption. With calcium and magnesium carbonate added to the gastric juice appreciable losses of thiamine occur as a result of both alkaline destruction and adsorption. Losses of the vitamin because of the presence of aluminum hydroxide are slight and are

due entirely to adsorption. These interpretations for the loss of the added thiamine are based upon the comparative recovery figures obtained from similar samples adjusted to the same pH values with sodium hydroxide.

In Table II are presented our findings concerning the stability of thiamine in hemin-containing gastric juice and in gastric juice in achlorhydria. In the former case, normal gastric juice was used with the added blood having had its cells previously laked by dilution with water. In the latter case the gastric juice was obtained directly from patients with achlorhydria; the reactions of the samples were not adjusted. The vitamin was found to be

TABLE III

Fate of Thiamine in Hepatic Bile

Sample (50 cc.)	pH of incu-bated material	Thiamine added	Temperature during 16 hr. incubation	Apparent thiamine in sample	Apparent loss of added thiamine*
		γ	°C.	γ	per cent
A	4.5	0	Immediate determination	<5	
B	4.5	100	" "	104	0
C	8.0	30	37.5	12	65
D	8.0	100	37.5	50	52
E	8.0	5000	37.5	1100	78
F	4.5	100	37.5	101	3

* For these calculations 4 γ of thiamine were assumed to be present initially in the 50 cc. bile sample.

stable in the presence of hemin[5] and in gastric juice in achlorhydria. The small amounts of thiamine lost during some of the tests can be entirely attributed to the alkalinity of the solutions.

Fate of Thiamine in Hepatic Bile—In the present study eleven different bile samples, obtained from patients during the last days of postoperative T-tube drainage, were used. The reaction of such hepatic bile varies from pH 8.0 to 8.5.

The initial studies summarized in Table III indicate that the

[5] Additional tests were carried out with 2.5 cc. of blood (the cells having been previously laked) added to 6 hour aliquots of pooled freshly voided urine samples. The pH of the solutions was adjusted to from 4.5 to 8.0 and the thiamine then added. In no case was there any loss of the vitamin during the following 6 hour incubation period at 37.5°.

chemical method is applicable to the estimation of thiamine in bile. When thiamine is incubated with bile at its natural pH, there is a marked, apparent loss of the vitamin. The loss is not absolute but ranges from 50 to 90 per cent of the added thiamine, in this particular series from 52 to 78 per cent. During the period of incubation at pH 4.5 no loss of the vitamin occurs.[6]

Two factors may explain the apparent loss of thiamine, (a) actual destruction and (b) conversion of the vitamin to a compound which is not estimated by the chemical method. Such compounds, still possessing biological activity (13), are the monophosphoric and the pyrophosphoric ester (cocarboxylase) of thiamine. However, these esters can be quantitatively converted to free thiamine for chemical analysis by enzymic hydrolysis (12).

In the present study aliquots of the bile after the initial incubation periods were adjusted to pH 4.5. 1 gm. quantities of a yeast phosphatase powder (12) were added to 25 cc. samples of the test materials and the suspensions incubated at 45° for a period of at least 24 hours.[7] Thiamine was then determined (5, 12). Representative data are presented in Table IV. Because of the considerable amount of thiamine in the yeast enzyme preparation (170 γ per gm.), large quantities of thiamine (5000 γ) were added to the 50 cc. bile specimens. In this way the amount of thiamine added in the yeast powder is rendered relatively insignificant, so that possible errors, associated with the use of a difference method, would be negligible.

The stability of thiamine in bile at pH 4.5 is again noted. An apparent loss of 88 per cent of the added vitamin is observed when the bile is incubated at its natural pH (Sample B). Subsequent incubation of an aliquot of this same sample with the yeast enzyme powder (Sample D) is followed by a recovery of 56 per cent

[6] The pH values of the bile samples remain practically constant during the periods of incubation.

[7] Preliminary control tests with added synthetic and naturally occurring phosphorylated thiamine, similar to those carried out in our study of the form in which the vitamin is excreted in urine (5), indicated that no inhibitory effect is exerted by bile upon the enzymic conversion of phosphorylated to free thiamine. Synthetic cocarboxylase was kindly furnished by Merck and Company, Inc., Rahway, New Jersey. The naturally occurring phosphorylated vitamin was obtained from a hot air-dried yeast powder (12).

of the originally added thiamine, 44 per cent more than the 12 per cent initially found.   This is the maximal recovery value

TABLE IV

Destruction of Thiamine and Cocarboxylase by Bile at Its Natural pH;
Conversion of the Vitamin into a Complex Which Is Determined
Chemically Only after Incubation with Yeast Enzymes

Sample	Experiment*	Free thiamine found†	
		γ	per cent
A	Bile at pH 4 5 + 5000 γ thiamine, 37.5°, 16 hrs.	5020	100
B	" " " 8 1 + 5000 γ " 37.5°, 16 "	620	12
C	As in B, then incubated at pH 4.5, 45°, 24 hrs.	720	14
D	" " " " " with yeast powder at pH 4.5, 45°, 24 hrs.	2800‡	56
E	As in B; then incubated with yeast powder at pH 4 5, 45°, 48 hrs.	2700‡	54
F	Aqueous solution of cocarboxylase ($\approx$ 1000 γ thiamine) buffered at pH 8.1, 37.5°, 16 hrs.	0	0
G	Aqueous solution of cocarboxylase ($\approx$ 1000 γ thiamine) incubated with yeast powder at pH 4.5, 45°, 24 hrs.	960‡	96
H	Bile at pH 8.1 + cocarboxylase ($\approx$ 1000 γ thiamine) 37.5°, 16 hrs.	160	16
I	As in H; then incubated with yeast powder at pH 4.5, 45°, 24 hrs.	550‡	55
	Tests for cocarboxylase activity	Cocarboxylase found§	
J	Bile at pH 8.1 + 5000 γ thiamine, 37.5°, 16 hrs.	0	0
K	" " " 8.1, 37.5°, 16 hrs.; 1000 γ cocarboxylase then added followed by immediate determination	1000	100
L	Bile at pH 8.1 + 1000 γ cocarboxylase, 37.5°, 16 hrs.	0	0

* In each case 50 cc. aliquots of the same bile sample were used.

† Free thiamine was determined by the chemical method based upon the reaction between the vitamin and diazotized *p*-aminoacetophenone (5, 12).

‡ These values have been corrected for the thiamine in the yeast powder used for the enzymic conversion of the combined forms to the free vitamin.

§ Cocarboxylase was determined by the Lohmann and Schuster (13) manometric method based upon the enzymic decarboxylation of pyruvic acid.

(Sample E).   That an enzyme in the yeast powder is responsible for the increased yield of free thiamine is indicated by the low

recovery in Sample C, when an aliquot of the test material is incubated under the same conditions as in the case of Samples D and E but this time without the yeast enzyme preparation. Apparently the true loss of thiamine, following its incubation with this particular bile sample at its natural pH, is 45 per cent.

The source of the extra thiamine liberated enzymically was at first interpreted as being the phosphorylated vitamin (1). Further studies have indicated that such is not the case. The chemical analyses of Samples F, G, and H (Table IV) show that bile during its incubation with phosphorylated thiamine (cocarboxylase in these tests) may actually exhibit a phosphatase activity. The conversion of as much as 16 per cent of the added cocarboxylase to free·thiamine under such circumstances seems to indicate that no appreciable phosphorylation of thiamine can occur during its incubation with bile. Synthetic cocarboxylase (Sample I) and naturally occurring phosphorylated thiamine (unpublished experiments) appear to be destroyed to the same extent as the free vitamin when incubated with bile at its natural pH.

Enzymic tests (13) for one of the phosphoric acid esters of thiamine, cocarboxylase, have shown that the added vitamin is not converted to this form (Sample J). Indeed, cocarboxylase activity *completely* disappears when the ester is incubated with the bile at its natural pH (Sample L). The control test (Sample K) indicates that this loss of cocarboxylase activity is real and not due to an inhibition of the enzymic reaction, decarboxylation of pyruvic acid, by the presence of bile.[8]

In Table V are summarized the results of studies of the influence of enzyme inhibitors upon the conversion of free thiamine to the unknown complex during its incubation with bile. If an enzyme is involved in this conversion, it must be heat-stable. Repeated tests, comparable to those carried out with Sample C, have shown that preliminary heating of the bile at 100° for a period of 30 minutes at pH 4.5 which approaches the isoelectric point of most proteins, fails to inhibit the conversion. Sodium iodoacetate is likewise ineffective in a concentration twice that which completely inhibits the phosphorylation of thiamine (14). The absolute amount of the unknown complex formed is appreci-

ably less in the presence of the iodoacetate but because this salt destroys thiamine at an alkaline pH less of the vitamin remains for the conversion.[9] Thus, the fraction so changed, when expressed in terms of total thiamine remaining after the period of incubation, is no less than that observed in the absence of the enzyme inhibitor. Chloroform completely inhibits the conversion of thiamine into the unknown complex.[10]

TABLE V

Influence of Enzyme Inhibitors upon Conversion of Free to Combined Thiamine during 16 Hour Incubation of the Vitamin with Bile at 37.5°

Sample	Enzyme inhibitor	pH of incubated material*	Thiamine in sample		Fate of added thiamine		
			Free	Total†	Apparent loss	Combined‡	True loss
			γ	γ	per cent	per cent	per cent
A	None	4.5	5060	5040	0	0	0
B	"	8.0	1200	2180	76	20	56
C	Heat§	8.0	2040	3200	59	23	36
D	Iodoacetate‖	8.0	300	1060	94	15	79
E	Chloroform¶	8.0	3460	3400	31	0	32

* In each case 5000 γ of thiamine were added to 50 cc. of the bile after the pH of the latter had been adjusted to the desired value.

† Determined after incubation with the active yeast powder used for the enzymic hydrolysis of phosphorylated thiamine.

‡ Thiamine converted into a complex which is not determined chemically until liberated as the free vitamin by one of the yeast enzymes.

§ In this case the bile sample was first adjusted to pH 4.5 and then heated in a boiling water bath for 30 minutes. The original pH was restored, the vitamin added, and the solution incubated.

‖ Sodium iodoacetate was added to the bile to make a 0.01 M solution.

¶ 2 cc. of chloroform were added to the bile.

[9] Because of the marked instability of the vitamin during its incubation in pure aqueous solution at a slightly alkaline reaction, it is impossible to study directly the destructive action of sodium iodoacetate. However, in gastric juice the vitamin is relatively stable at pH 8.0 (Table I). The addition to such a solution of sodium iodoacetate to make a concentration of 0.01 M is followed by a loss of about 70 per cent of the vitamin during the period of incubation. No loss of the added thiamine occurs at pH 4.5 in the presence of the same concentration of iodoacetic acid.

[10] In testing aliquots of the incubated bile sample in this case the pH was first adjusted to 4.5 and the chloroform removed by repeated *in vacuo* distillations. The yeast enzyme preparation was then added.

Additional tests summarized in Table VI were carried out to determine the fate of thiamine in bile when incubated at different pH values for varying periods of time. The conditions observed in these tests bracket the physiological range in both normal and pathological organisms. The results obtained indicate that appreciable destruction of the vitamin occurs even during the short periods of incubation. As the pH of the bile is made acid, less thiamine is lost. The percentage conversion of the residual vitamin into the unknown complex increases with the

TABLE VI

Stability of Thiamine in Hepatic Bile Incubated at 37.5° at Different pH Values for Varying Periods of Time

Sample	pH of incubated material*	Period of incubation	Thiamine in sample		Fate of added thiamine		
			Free	Total†	Apparent loss	Combined‡	True loss
		hrs.	*γ*	*γ*	*per cent*	*per cent*	*per cent*
A	4.5	16	5060	5000	0	0	0
B	6.5	4	4000	4320	20	6	14
C	8.3	4	2540	3600	49	21	28
D	6 5	8	3460	4120	31	13	18
E	8.3	8	2060	3260	59	24	35
F	6.5	16	2400	3580	52	24	28
G	8.3	16	1030	2520	79	29	50

* In each case 5000 γ of thiamine were added to 50 cc. of the bile after the pH of the latter had been adjusted to the desired value.

† Determined after incubation with the active yeast powder used for the enzymic hydrolysis of phosphorylated thiamine.

‡ Thiamine converted into a complex which is not determined chemically until liberated as the free vitamin by one of the yeast enzymes.

alkalinity of the solution. The same results are obtained with bile, stored at 4° for a period of 1 week. With more dilute solutions of thiamine in bile (1.0 γ per cc.) comparable findings are also noted.

Fate of Thiamine in Duodenal Pancreatic Juice—Three samples of duodenal pancreatic juice[11] were obtained by suction through a 2-way gastroduodenal tube from two subjects; in one case the

[11] The authors are indebted to Dr. H. M. Pollard of the Department of Medicine, University of Michigan, for the collection of these samples.

specimens were collected before and after secretin stimulation; in the other case, only after the injection of the hormone. All samples contained bile. When the specimens were incubated with added thiamine at their natural pH of approximately 8.1, the same percentage destruction and conversion of thiamine to the unknown complex as were noted in the tests with hepatic bile were observed.

DISCUSSION

Only *in vitro* experiments have been reported in the present study. This point is emphasized because thiamine is more stable in its natural habitat than in pure aqueous solutions. Whenever complete recovery of added thiamine is reported, it can be safely assumed that no destruction would occur under comparable circumstances *in vivo*. On the other hand, the losses *in vivo* are believed to be appreciably less than those noted in the present investigation, owing to the protective action of the other constituents of the diet. This protective action is known to operate in so far as loss of the vitamin, both by adsorption and alkaline destruction, is concerned. However, even though the losses of the vitamin are not nearly as great *in vivo*, such losses are of physiological importance because of the fact that the average American diet is close to the border line of adequacy with respect to thiamine intake (15). The experimental lowering of the urinary thiamine values of the normal subject subsisting on a standardized adequate diet by concomitant ingestion of alkaline salts (unpublished experiments in our laboratory) indicates that the mechanisms responsible for the losses of thiamine *in vitro* do operate *in vivo*. Certainly the information derived from the present study may be applied directly to explain the relatively small urinary thiamine values obtained when the normal subject in the postabsorptive state takes the oral test dose of thiamine in pure aqueous solution (2).

From the tests reported two factors, over and above the direct protective action of the other dietary constituents, appear to operate in the normal organism to conserve the vitamin. Because of the presence of food in the stomach, the vitamin is retained for a longer period in that organ with no possible destruction occurring there. Accordingly, any thiamine absorbed directly

from the stomach would not be subjected to the destructive factors in the small intestine.   Secondly, the normal acidity of the gastric chyme reduces the pH of the secretions in the duodenum and this in turn decreases the possible destruction of the vitamin by bile and pancreatic juice.

The destruction of thiamine by gastric juice in achlorhydria reported by Sinclair and coworkers (9–11) may be due entirely to contamination of the samples with bile.   Only with extreme care can such gastric juice be collected free from bile.   It is quite possible that the results of our tests with duodenal pancreatic juice are also entirely due to the presence of bile in the samples.

Because of the demonstrated remarkable specificity of the chemical method for the intact thiamine molecule (5, 12), we have interpreted the increased values obtained subsequent to the incubations with the yeast enzyme preparation as due to free thiamine liberated from some complex.   The validity of this interpretation can be tested only by properly controlled biological assay of the bile sample after incubation at its natural pH with thiamine.   With respect to the specificity of the method for thiamine, it is worth placing on record four additional tests to supplement those already reported (5, 12).   Sulfathiazole, sulfamethylthiazole, dihydrothiamine, and dihydrococarboxylase fail to give positive tests; none of these compounds has biological activity in animals (16–18).

SUMMARY

1. Thiamine is stable in normal gastric juice from pH 1.5 to 8.0 during a 16 hour incubation at 37.5°.

2. In the presence of antacids thiamine added to gastric juice may be adsorbed or destroyed during the period of incubation.

3. Thiamine is stable in hemin-containing gastric juice and in gastric juice from patients with achlorhydria.

4. When thiamine is incubated for 16 hours with bile at its natural pH, there is an apparent loss of from 50 to 90 per cent of the vitamin.   Subsequent incubation with a yeast enzyme preparation results in the recovery of more thiamine; the true loss then varies from 40 to 55 per cent of the added vitamin.   The precursor of the extra thiamine liberated enzymically is not the phosphorylated vitamin.   The influence of enzyme inhibitors, pH,

and time of incubation upon the conversion of thiamine into this unknown complex is reported. As the reaction of the bile becomes acid, less thiamine is destroyed with complete recoveries obtained from samples incubated at pH 4.5.

5. The results obtained when thiamine is incubated with pancreatic juice are similar to those noted with bile.

6. The *in vivo* implications of these *in vitro* studies are discussed.

BIBLIOGRAPHY

1. Melnick, D., Robinson, W. D., and Field, H., Jr., *Proc. Am. Soc. Biol. Chem.*, *J. Biol Chem.*, **133**, p. lxvii (1940).
2. Melnick, D., Field, H., Jr., and Robinson, W. D., *J. Nutrition*, **18**, 593 (1939).
3. Robinson, W. D., Melnick, D., and Field, H., Jr., *J. Clin. Inv.*, **19**, 399 (1940).
4. Diet manual of University Hospital, Ann Arbor (1940).
5. Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, **130**, 97 (1939).
6. Mahlo, A., *Deutsch. med. Woch.*, **64**, 406 (1938).
7. Schroeder, H., *Klin. Woch.*, **18**, 148 (1939).
8. Leong, P. C., *Biochem. J.*, **31**, 1391 (1937).
9. Sinclair, H. M., *Proc. Roy. Soc. Med.*, **32**, 807 (1939).
10. Laurent, L. P. E., and Sinclair, H. M., *Lancet*, **1**, 1045 (1938).
11. Goodhart, R., and Sinclair, H. M., *J. Biol. Chem.*, **132**, 11 (1940).
12. Melnick, D, and Field, H., Jr., *J. Biol. Chem.*, **127**, 515, 531 (1939).
13. Lohmann, K., and Schuster, P., *Biochem. Z.*, **294**, 188 (1937).
14. Ochoa, S., *Biochem. J.*, **33**, 1262 (1939).
15. Cowgill, G. R., *J. Am. Med. Assn.*, **111**, 1009 (1938).
16. Williams, R. R., and Spies, T. D., Vitamin B_1 (thiamin) and its use in medicine, New York (1938).
17. Lipmann, F., and Perlmann, G., *J. Am. Chem. Soc.*, **60**, 2574 (1938).
18. Stern, K. G., and Melnick, J. L., *J. Biol. Chem.*, **131**, 597 (1939); **135**, 365 (1940).

THE INFLUENCE OF ALKALOSIS AND ACIDOSIS UPON FASTING KETOSIS

By EATON M. MacKAY, ARNE N. WICK, HERBERT O. CARNE, AND CYRUS P. BARNUM

(*From the Scripps Metabolic Clinic, La Jolla, California*)

A normal mechanism for utilizing fatty acids which is brought into play during fasting consists of a partial oxidation of these substances to form ketone bodies by the liver and distribution of these ketone bodies to the tissues by the blood for complete combustion (1–3). We refer to the presence of measurable amounts of ketone bodies in the blood as a ketosis. The ketogenic action of an alkalosis during fasting is well known (4–9). That an acidosis on the other hand may be antiketogenic is not so generally recognized. Adlersberg (10) found that acid ammonium phosphate, which causes an acidosis, diminishes the ketonuria of starvation. It was the purpose of this study to investigate the influence of alkalosis and acidosis upon ketosis in the fasting albino rat and attempt to determine the mechanism whereby these conditions affect ketosis.

The methods used were similar to those applied before (11, 12) to studies of ketosis. Our early experiments were carried out before a satisfactory blood ketone method (13) for small samples of blood had been developed. The variation in the ketonuria was observed. Experiment 1 (Table I) is a typical example. An acidosis produced by feeding 0.2 N HCl decreased the excretion of ketone bodies in the urine, while $NaHCO_3$ administration was followed by an increase in the ketonuria. With the availability of blood ketone body determinations this effect was confirmed by following the changes in the blood level as a measure of ketosis. The ketogenic effect of alkali and the antiketogenic influence of acid are shown during a single day in Experiment 2 (Fig. 1). It is just as obvious when the blood ketone level is

followed from day to day in rats receiving acid and alkali (Experiments 3 and 4, Table II). In Experiment 4 the blood samples were obtained only 3 hours after acid and alkali had been fed but in Experiment 3 from 6 to 8 hours had elapsed.

The difference in the degree of ketosis in fasting as measured by the blood ketone level of fasting rats of the control groups (Experiments 3 and 4, Table II) is interesting. The lower levels (Experiment 4) were obtained during the summer months, when we find that these values tend to be low. We have an idea that

TABLE I

Influence of Acidosis Produced by Feeding HCl and Alkalosis Produced by Feeding NaHCO₃ upon Ketonuria, Urine Nitrogen, and Body Glycogen of Fasting Rats (Experiment 1)

Group	Urine ketone bodies per sq. dm. body surface per day				Urine N per sq. dm. body surface per day				Glycogen at end of fast	
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Liver	Total body
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg. per rat*	*mg. per rat*
Acid-fed	8	22	15	9	34	31	27	24	59	255
Control.	34	49	54	34	26	23	24	18	34	196
Alkali-fed	82	63	75	42	21	16	15	15	20	173

There were five male rats in each group with an average body weight for each group of 170 gm. Prior to fasting the rats had been receiving a diet low in protein which tends to produce a high ketonuria on subsequent fasting (14). Observations were commenced on the 3rd day of fasting. During the ensuing 4 days the control group was given 0.2 N NaCl, the acid-fed group 0.2 N HCl, and the alkali-fed group 0.2 N NaHCO₃ in doses of 1 cc. per sq. dm. of body surface three times each day.

this variation is connected with body protein storage and the differences in the daily urine nitrogen excretion between the control groups of Experiments 3 and 4 (Table II) would favor such an explanation.

The presence of a ketosis during fasting depends upon a relative lack of carbohydrate. The ketosis will vary inversely with the amount of carbohydrate available. The organism subsists upon stored fat and tissue (and "stored") protein after the meager glycogen stores are gone. Since glucose is antiketogenic, and if enough is available for use there is no ketosis, we presume that

the amount of fat converted to ketone bodies during fasting will depend largely upon the amount of glucose formed from protein (and the glycerol of fat).   An alteration in the rate of catabolism of protein and hence the formation of glucose from protein appears to be the mechanism whereby acidosis and alkalosis affect ketosis.
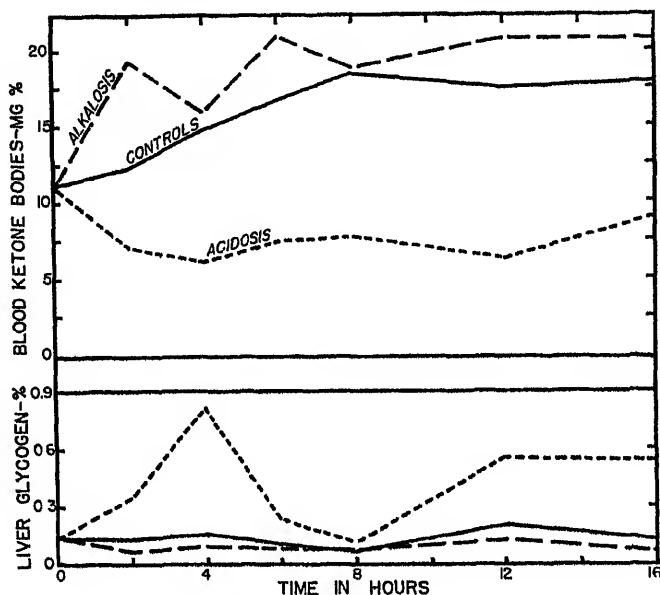
Fig. 1. Influence of acidosis and alkalosis upon the blood ketone body and liver glycogen concentration during a single day (Experiment 2). The observations were made on the 3rd day of fasting, the rats having previously been upon the stock diet.   The control group was given 1 cc. of 0.6 N NaCl and the alkali-fed group 1 cc. of 0.6 N NaHCO₃ per sq.dm. of body surface by stomach tube at the 0 hour.   The acid-fed groups received a similar dose of 0.2 N HCl at 0, 4.5, and 6.5 hours.   Each observation recorded was obtained by sacrificing a group of five female rats, 4 months old, with an average body weight of 155 gm.

We have evidence for such variations in glucose formation—increased liver glycogen after acid and decreased liver glycogen after alkali—in all of our experiments.   That this is a result of the changes in the rate of protein catabolism, shown by a rise in the daily urine nitrogen excretion with acid administration and a fall when alkali is given, seems evident from the urine nitrogen

Table II

Influence of Acidosis and Alkalosis upon Blood Ketone Body Concentration, Liver Glycogen Concentration, and Daily Excretion of Nitrogen and Sulfur in Urine

Experiment No.	Group	Blood ketone bodies					Liver glycogen concentration					Urine N per sq. dm. body surface per day					
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent	per cent	per cent	per cent	per cent	mg.	mg.	mg.	mg.	mg.	mg.
3*	Acid-fed		11.8		11.4	5.2		0.33		0.63	0.78	41	31	28	30	38	39
	Control		20.8		23.3	10.1		0.23		0.23	0.52	26	24	23	22	22	21
	Alkali-fed		26.0		26.1	15.6		0.09		0.22	0.30	23	22	20	20	21	17
4†	Acid-fed	4.2	6.2	5.8	6.6		0.17	0.39	1.38	0.96		29	32	35	35		
	Control	10.0	11.7	8.6	10.8		0.06	0.26	0.67	0.75		26	30	33	22		
	Alkali-fed	12.2	15.1	11.0	13.3		0.09	0.06	0.25	0.41		21	26	30	17		

	Group	Urine S per sq. dm. body surface per day			
		Day 1	Day 2	Day 3	Day 4
		mg.	mg.	mg.	mg.
	Acid-fed	0.065	0.075	0.060	0.074
	Control	0.063	0.065	0.051	0.067
	Alkali-fed	0.060	0.062	0.052	0.063

* Observations on male rats averaging 237 gm. in weight. After having been on the stock diet, they were fasted and observations begun on the 2nd day of fasting. The doses of acid, alkali, and sodium chloride were the same as in Experiment 2 except that only two doses were given each day. There were twelve rats in each group and four from each group were sacrificed to obtain the blood ketone body and liver glycogen concentrations. Consequently the urine collections on Days 1 and 2 were made on twelve rats, on Days 3 and 4 on eight rats, and on Days 5 and 6 on four rats.

† Male rats averaging 202 gm. in weight were used and treated as in Experiment 3. There were twelve rats in each group and three were sacrificed at the end of each day.

figures in Experiments 1 (Table I), 3, and 4 (Table II).   The variations in sulfur excretion in the urine (Experiment 4, Table II) are added proof of the alteration in protein catabolism.

The evidence presented here that an acidosis produced by a mineral acid is antiketogenic during fasting because of the increased protein → glucose is somewhat stronger than the reverse in the case of alkalosis.   However, a decrease in the catabolism of protein → glucose is at present by far the most reasonable explanation of the mechanism by which alkali exerts a ketogenic action.   Haldane (6) expressed the opinion that the ketonuria of alkalosis is due to a deficient carbohydrate metabolism but he thought that an alkalosis reduced the ability of the organism to utilize sugar.

SUMMARY

An acidosis produced by feeding HCl is antiketogenic and an alkalosis resulting from $NaHCO_3$ administration is ketogenic in the fasting albino rat when ketosis is measured by the level of the ketone bodies in the blood as well as the degree of ketonuria. Acid administration causes an increase in the liver glycogen concentration and in the daily excretion of nitrogen and sulfur in the urine, while the alkalosis leads to a decrease from normal in all of these values.   It is concluded from this that the antiketogenic effect of acidosis depends upon an increase in protein catabolism and an increase in glucose formation therefrom, while alkalosis is ketogenic because it has an opposite effect upon this process.

BIBLIOGRAPHY

1. Barnes, R. H., and Drury, D. R., *Proc. Soc. Exp. Biol. and Med.*, **36**, 350 (1937).
2. Blixenkrone-Möller, N., *Z. physiol. Chem.*, **253**, 261 (1938).
3. Barnes, R. H., Drury, D. R., Greeley, P. O., and Wick, A. N., *Am. J. Physiol.*, **130**, 144 (1940).
4. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L., *J. Physiol.*, **54**, 32 (1920).
5. Porges, O., and Lipschütz, H., *Arch. exp. Path. u. Pharmakol.*, **97**, 379 (1923).
6. Haldane, J. B. S., *Lancet*, **1**, 537 (1924).
7. Booher, L. W., and Killian, J. A., *Proc. Soc. Exp. Biol. and Med.*, **21**, 528 (1924).
8. Beumer, H., and Soccknick, A., *Z. Kinderh.*, **37**, 236 (1924).

9. Butts, J. S., Deuel, H. J., Jr., and Hallman, L., *Proc. Soc. Exp. Biol. and Med.*, **32,** 898 (1934–35).
10. Adlersberg, D., *Biochem. Z.*, **143,** 527 (1923).
11. MacKay, E. M., Sherrill, J. W., and Barnes, R. H., *J. Clin. Inv.*, **18,** 30 (1939).
12. MacKay, E. M., Carne, H. O., and Wick, A. N., *J. Biol. Chem.*, **133,** 59 (1940).
13. Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, **131,** 413 (1939).
14. MacKay, E. M., Carne, H. O., and Wick, A. N., *Proc. Soc. Exp. Biol. and Med.*, **41,** 40 (1939).

THE RÔLE OF NICOTINAMIDE AND RELATED COMPOUNDS IN THE METABOLISM OF CERTAIN BACTERIA*

By FELIX SAUNDERS, ALBERT DORFMAN, AND
STEWART A. KOSER

(From the Departments of Biochemistry and of Bacteriology and Parasitology
of the University of Chicago, Chicago)

In a previous publication we have reported the isolation of nicotinamide and nicotinic acid from pig spleen and have demonstrated the importance of these compounds for the growth of a large number of strains of dysentery bacilli (1). The necessity of nicotinamide for the growth of a number of other organisms has been shown by other workers (2).

The essential nature of nicotinic acid and its amide has generally been attributed to the need of these compounds for synthesis of diphosphopyridine nucleotide and triphosphopyridine nucleotide. Thus Fildes has demonstrated that *Proteus vulgaris* grown on a medium containing nicotinic acid synthesizes factor V required for the growth of *Hemophilus influenzae* and *Hemophilus parainfluenzae* (3). We have demonstrated the synthesis of factor V by certain strains of dysentery bacilli (1). The only compounds of known composition which have factor V activity are the two nicotinamide-containing coenzymes.[1]

If the nicotinamide-containing coenzymes account for the only physiological rôle of nicotinamide, then it is to be expected that either one or both of these compounds should be at least as effi-

[1] Bass and others working in this laboratory have recently shown that catalase is able to act as factor V. They suggest that this effect may be due to some nicotinamide derivative other than the pyridine nucleotides.

cient and probably more efficient than nicotinamide in promoting the growth of dysentery bacilli. In a recent paper we have shown that, on the contrary, nicotinamide is more active than either of the pyridine nucleotides (4) and, furthermore, the growth-promoting activity of both of the coenzymes can be increased by hydrolysis.

These results led us to suspect that nicotinamide may be used in the metabolism of these organisms in some way other than for the synthesis of either of the pyridine nucleotides.

Lwoff and Lwoff (5) found that the rate of decolorization of methylene blue in the Thunberg tube by *Hemophilus parainfluenzae* can be increased by the addition of factor V (diphosphopyridine or triphosphopyridine nucleotide) provided the cells are grown on a medium deficient in factor V.

We have shown that within certain limits the growth of dysentery bacilli is proportional to the concentration of nicotinamide in the medium. We have utilized this property to prepare cells which can be stimulated not only by the addition of diphosphopyridine or triphosphopyridine nucleotide but also by nicotinic acid and certain of its derivatives.

Methods

Cultures—Shigella paradysenteriae, Sonne type (Strain 8), was originally isolated from a case of dysentery. The cultural and biochemical characteristics are those of the Sonne type and the cells are agglutinated by Sonne antiserum. This strain has been under laboratory cultivation for about 3 years. The *Proteus vulgaris* culture is a stock laboratory strain showing typical cell morphology and fermentation tests.

*Medium—*The following synthetic medium was used to grow all cells employed in these experiments: $(NH_4)_2HPO_4$ 2.0 gm., KH_2PO_4 1.5 gm., NaCl 5.0 gm., $MgSO_4$ 0.1 gm., redistilled water 1000 cc. The pH is 6.9. After autoclave sterilization sufficient sterile glucose solution is added to give a final concentration of 0.25 per cent. Finally, the requisite amount of nicotinamide is added from a sterile solution. In most experiments 0.006 or 0.010 γ per cc. of nicotinamide was used.

*Preparation of Cells—*The flask of synthetic medium is inoculated from a light suspension of cells in a buffered salt solution.

This suspension is made by adding 0.1 cc. of a 24 hour broth culture to 5 cc. of the buffered salt solution and 0.1 cc. of the resulting suspension is used for inoculation of each 100 cc. of medium.   24 hours were sufficient to obtain a maximum growth of dysentery bacilli on this medium but it was necessary to wait 48 hours to obtain consistent maximum growth of *Proteus*.   The cells were used at the end of these times.   The cells are collected by centrifuging for 1 hour in the freezing room ($-4°$).   The supernatant medium is discarded and the cells are suspended in about 40 cc. of 0.05 M KH_2PO_4 buffer of pH 7.4.   This suspension is then centrifuged and the cells are washed once more with phosphate buffer.   The twice washed cells are finally suspended in 26 cc. (this quantity varied sometimes with the particular experiment and the amount of cells used) of the phosphate buffer and are then ready for use.

Measurement of Oxygen Uptake—The measurement of the oxygen uptake was made in the presence of air by the direct Warburg method.   The glucose and the cell suspension were placed in the main vessel and the substance being tested for coenzyme activity was placed in the side arm and tipped in after temperature equilibration.   0.3 cc. of a 2 per cent glucose solution is added to each flask; the total volume of the contents of each flask is 2.7 cc.

If cells are used which were grown on a medium containing less than 0.02γ of nicotinamide per cc., the rate of reduction of methylene blue in the Thunberg tube is greatly accelerated by the addition to the tube not only of diphosphopyridine nucleotide, but also by the addition of nicotinic acid, its amide, and certain other derivatives (6).   If we consider the ratio of the time of reduction of the control to the time of reduction with added nicotinamide, we find that this ratio decreases as the amount of nicotinamide in the *culture medium* increases.   After a certain concentration (about 0.020γ per cc.) of nicotinamide in the medium is reached, the stimulating effect of nicotinamide (or coenzyme) added to the Thunberg tube disappears.   As the amount of nicotinamide in the medium is decreased, the cells become more responsive to the action of added nicotinamide, until the amount of nicotinamide present as growth factor is too small to obtain an adequate crop of cells.

Coenzyme Activity of Nicotinamide and Related Compounds—
For most of these experiments cells grown in 400 cc. of medium
containing either 0.006 or 0.010 γ per cc. of nicotinamide were
used for twelve Warburg vessels. The quantity of cells used
in any particular experiment does not affect the results as ex-
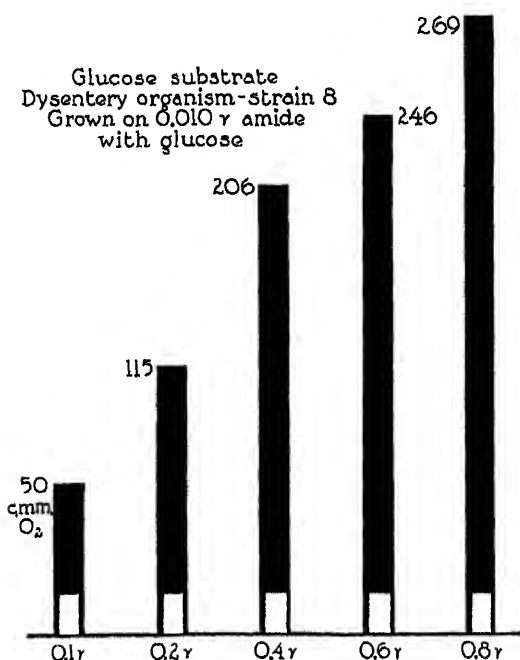
Fig. 1. Effect of nicotinamide on respiration. O_2 uptake for 2 hours.
The clear space indicates the respiration without the addition of nico-
tinamide.

pressed, since all comparisons of activity were made on the same
suspension at the same time.

When nicotinamide is added to cells grown on glucose under
the conditions described above, the respiration is very greatly
accelerated. Fig. 1 shows the effect of different concentrations
of nicotinamide on the respiration. The white space indicates
the respiration in the absence of nicotinamide, while the dark
space indicates the increase in oxygen uptake after the addition

of nicotinamide. This relation holds for concentrations from 0.1 to 0.8 γ per vessel (0.037 to 0.296 γ per cc.). The oxygen uptakes given in Fig. 1 represent the total uptake for a 2 hour period. Experiments at all concentrations were carried out in duplicate; each experiment was run at least twice and in most cases several times.

Fig. 2 illustrates the comparative activity of nicotinic acid and nicotinamide. It will be noted that nicotinamide is considerably more active than nicotinic acid. The relative results obtained
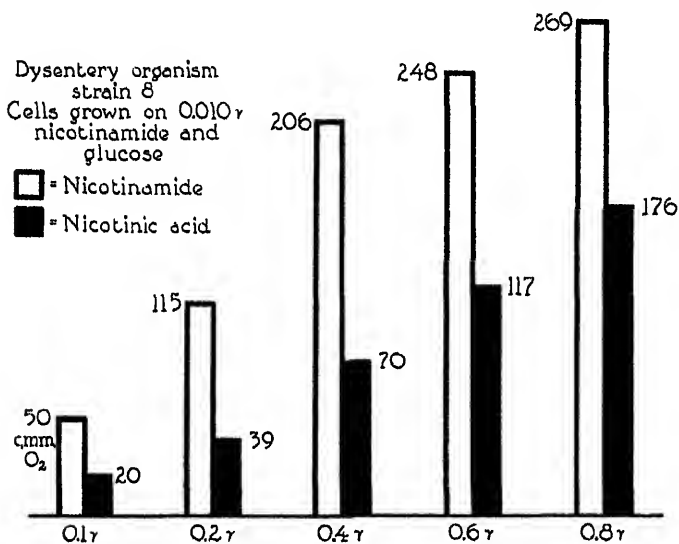
FIG. 2. Comparison of nicotinamide and nicotinic acid. O_2 uptake for 2 hours.

for these two compounds depend to some extent on the time elapsed after tipping in the accelerator. Nicotinamide is considerably more active during the early part of the run but as the experiment progresses the activity of the acid increases until it is almost equal to that of the amide.

We have previously reported (1) that the growth of dysentery bacilli in the presence of nicotinamide is more prompt than that supported by nicotinic acid. The comparative activities with respect to both the promotion of growth and the stimulation of respiration are consistent with the idea that nicotinic acid must

first be converted to nicotinamide before it can be utilized by the cell.

Fig. 3 indicates the relative activity of the various esters and nicotinamide which have previously (1) been shown to have a growth-promoting action on dysentery bacilli. It will be noted that methyl nicotinate is slightly more active than nicotinamide. The activities of the other esters are markedly lower. All substances used in this experiment were tested in equivalent concentrations. In repeated tests comparing methyl nicotinate and nicotinamide at a number of concentrations the methyl ester was
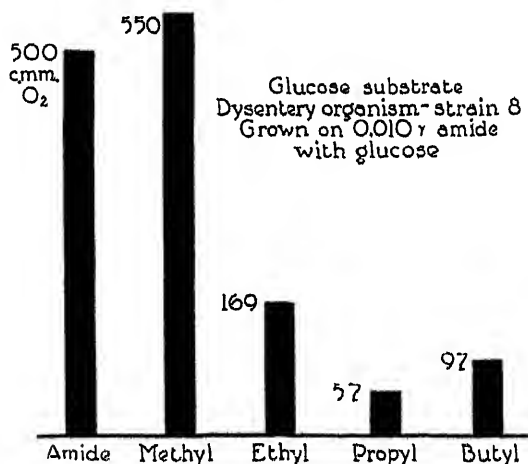
Fig. 3. Comparison of activity of amide and esters of nicotinic acid. O_2 uptake for 2 hours.

at least as active and probably slightly more active than an equivalent amount of nicotinamide.

The most likely explanation for the activity of methyl nicotinate, and also of nicotinamide, is probably that these compounds are able to stimulate respiration in some way other than or in addition to the formation of the known pyridine nucleotides.

Comparison of Nicotinamide and Pyridine Nucleotides—We have already reported (1) that nicotinamide is more active on a molar basis than either diphosphopyridine or triphosphopyridine nucleotide in promoting the growth of dysentery bacilli.

Fig. 4 indicates the activity of diphosphopyridine nucleotide

in stimulating the respiration of dysentery bacilli. The diphos-
phopyridine nucleotide preparation used in this experiment was
prepared in this laboratory by Dr. A. Bass and was approximately
40 per cent pure. The concentrations are so chosen that they are
equivalent to those of nicotinamide in Fig. 1. On the basis of
numerous experiments there can be no doubt that on a molar
basis nicotinamide is more active than diphosphopyridine nucleo-
tide. To assure ourselves that our conclusions were not in error
owing to faulty standardization of the coenzyme preparation, we
have studied the effect of hydrolysis on the activity of diphos-
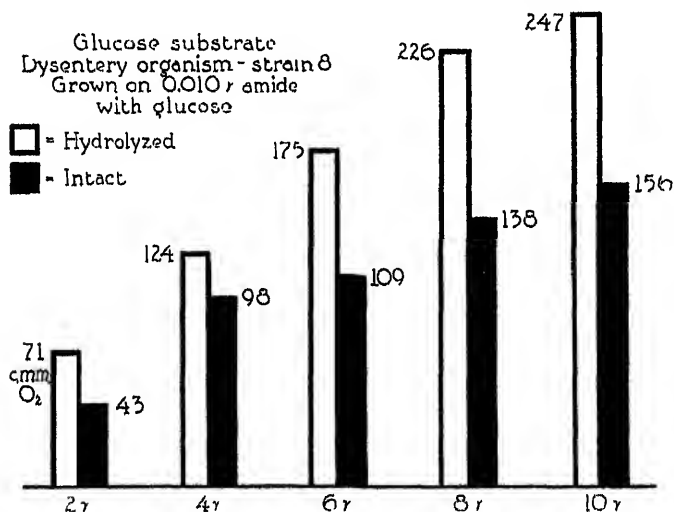
Fig. 4. Effect of hydrolysis on coenzyme I. O₂ uptake for 2 hours

phopyridine nucleotide. The hydrolysis was carried out by
autoclaving the coenzyme with 0.1 N H_2SO_4 for 1 hour at 20
pounds. Acid hydrolysis was used in preference to alkaline
hydrolysis to prevent conversion of the amide to the acid.

It will be seen in Fig. 4 that autoclaving diphosphopyridine
nucleotide increases activity, so that the activity of the hydrolyzed
sample is about the same as that of an equivalent amount of
nicotinamide. Apparently the free nicotinamide released by
autoclaving is more active than it is when combined in diphos-
phopyridine nucleotide.

The question then arises whether or not the compound that is actually utilized by these organisms is triphosphopyridine nucleotide.   Fig. 5 shows the activity of triphosphopyridine nucleotide.[2] The activity of intact triphosphopyridine nucleotide was found to be even lower than that of diphosphopyridine nucleotide but on hydrolysis the activity increased more markedly than in the case of diphosphopyridine nucleotide.
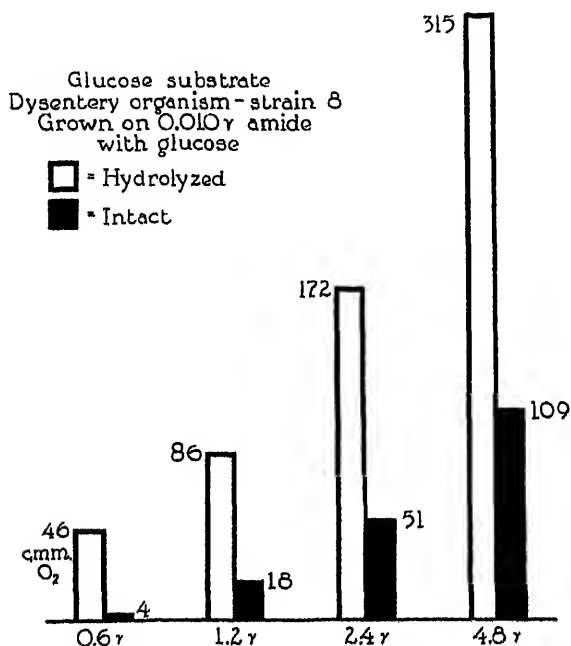
FIG. 5. Effect of hydrolysis on coenzyme II.   O₂ uptake for 2 hours

On the basis of these results it must be concluded that nicotinamide is more active than an equivalent amount of either diphosphopyridine or triphosphopyridine nucleotide. This finding becomes understandable if we assume that nicotinamide participates in the oxidation of glucose (or some product derived from glucose) in some way other than merely serving as a building block for one of the known pyridine nucleotides.

[2] The pure coenzyme preparation used in this experiment was obtained through the courtesy of Dr. Otto Warburg.

When *Proteus vulgaris* is grown on a medium deficient in nicotinamide, the cells have a very low respiratory rate with glucose as substrate. The addition of nicotinic acid, nicotinamide, methyl nicotinate, or intact or hydrolyzed diphosphopyridine or triphosphopyridine nucleotide results in a marked increase in oxygen uptake. Figs. 6 and 7 indicate the effect of these sub-
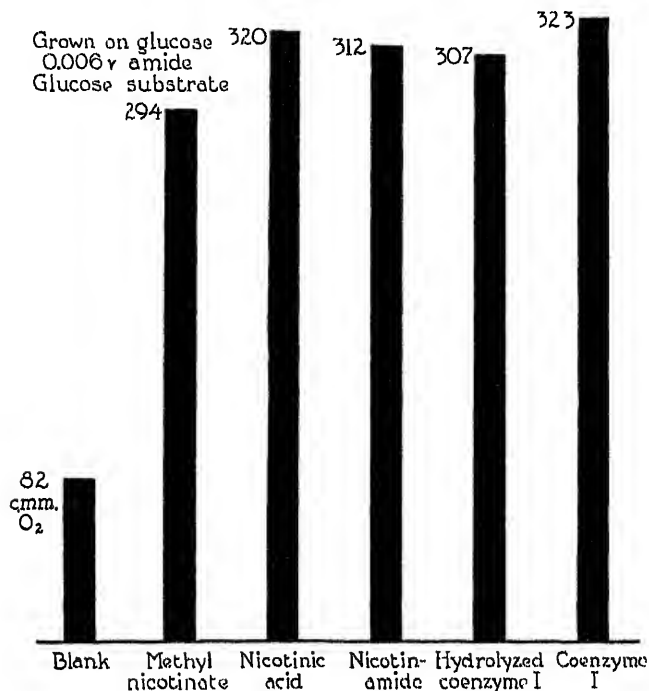
FIG. 6. Comparative activities for *Proteus vulgaris*. O_2 uptake for 2 hours

stances on the respiration of *Proteus vulgaris*. All of the substances were probably used in excess in this experiment, so that it is not possible to draw conclusions as to comparative activities on the basis of these data. The essential point is that all of the substances tested were active.

If now we use cells grown in exactly the same way with glucose as substrate but use lactate instead of glucose as substrate in the Warburg vessel, it is found that only diphosphopyridine nucleotide is active. Thus in Figs. 7 and 8 it is seen that when lactate is

used as a substrate, nicotinic acid, nicotinamide, methyl nicotinate, hydrolyzed diphosphopyridine nucleotide, triphosphopyridine nucleotide, and hydrolyzed triphosphopyridine nucleotide do not increase the respiration of these organisms, while intact diphosphopyridine nucleotide causes more than 100 per cent increase in the oxygen uptake. In this particular case the effect of diphosphopyridine nucleotide is quite specific. These results make
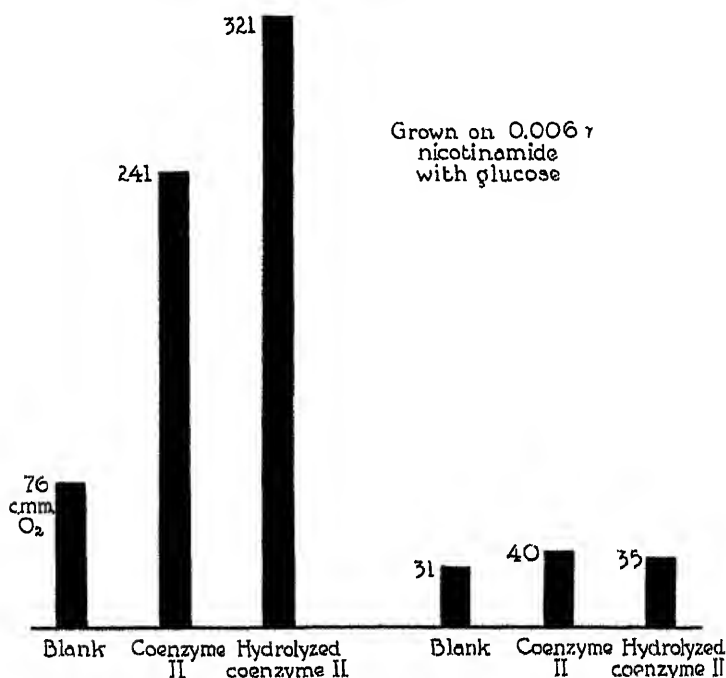
Fig. 7. Comparison of coenzyme II on lactate and glucose, for *Proteus vulgaris*. O_2 uptake for 2 hours.

it difficult to believe that the stimulation by nicotinamide can be explained on the basis of the rapid synthesis of coenzymes from nicotinamide.

We have also studied the ability of these organisms to oxidize several other substrates. The dysentery bacillus can oxidize lactate, glucose monophosphate, and glutamate. All the nicotinamide derivatives which stimulate the oxidation of glucose also stimulate the oxidation of these three substrates. *Proteus,*

however, behaves quite differently. The case of the oxidation of lactate by *Proteus* has already been discussed. When *Proteus* oxidizes glutamate, it behaves as it does toward lactate; that is, only intact diphosphopyridine nucleotide is able to stimulate the respiration. The strain of *Proteus* used by us is unable to oxidize glucose monophosphate in the presence of nicotinamide or any of its derivatives.
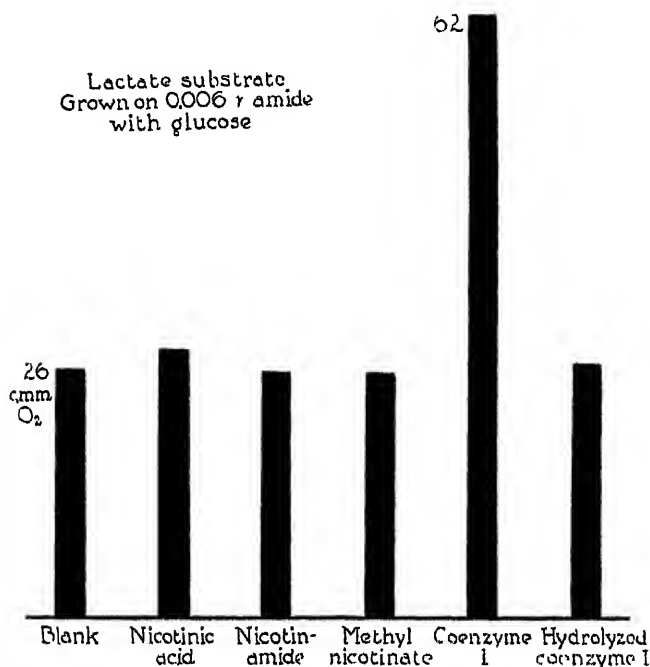
FIG. 8. Comparative activities for *Proteus vulgaris*. O$_2$ uptake for 2 hours

Barron[3] has found that nicotinamide forms a hemochromogen with blood hemin which shows the typical properties of these compounds of reversible oxidation and reduction. Nicotinic acid does not form a hemochromogen. It seems possible that the action of nicotinamide which we have observed in these experiments might be due to the formation of a hemochromogen which acts as a respiratory catalyst.

We have attempted to study this point but have obtained in-

[3] Barron, E. S. G., private communication.

conclusive results.   Table I shows the activity of a hemochromogen prepared from nicotinamide.   In all cases tried the hemochromogen was found to have the same activity as free nicotinamide. Such results neither prove nor disprove the possibility that nicotinamide might owe some of its activity to the formation of a hemochromogen. Thus, the cells might be saturated with hemin. In such a case the amount of nicotinamide present would be the determining factor and adding nicotinamide-hemochromogen would be equivalent to adding nicotinamide alone.

TABLE I

2 Hour Oxygen Uptake of Dysentery and Proteus Organisms Grown on 0.006 γ per Cc. of Nicotinamide

	Cells	Cells + 0 7 γ nicotinamide	Cells + 0.7 γ nicotinamide + 20 γ hemin	Cells + 20 γ hemin
	c.mm.	c.mm	c.mm.	c.mm.
Dysentery organism on glucose .	47	283	277	52
Proteus organism on glucose	102	250	246	106

DISCUSSION

The evidence presented above definitely indicates that nicotinamide is concerned in cellular oxidations in some way in addition to the formation of the pyridine nucleotides.   If one attempts to explain our results on the basis of diffusion, one must postulate that in the presence of glucose nicotinamide is able to diffuse into the cell and be synthesized to one of the phosphopyridine nucleotides more rapidly than an equivalent amount of the nucleotide can diffuse into the cell, while in the presence of lactate the free nicotinamide either cannot diffuse into the cell or cannot be synthesized to the coenzyme after entrance into the cell.

The fact that diphosphopyridine and triphosphopyridine nucleotides are less active than nicotinamide itself makes the interpretation that we are simply dealing with a synthesis of the coenzymes entirely unlikely, especially when we consider that autoclaving the coenzymes increases their activity.

For the present it seems simplest to hypothesize the formation of an unknown substance which can be synthesized from nicotinamide more readily than it can be formed from the known pyridine-containing coenzymes.   This unknown substance may be a

hemochromogen. The fact that hematin and coenzymes are both required by *Hemophilus influenzae* indicates a possible connection between the function of these two substances in the metabolism of this organism at least.

McIlwain has recently studied the action of pyridine-3-sulfonic acid and its amide as inhibitors of bacterial growth (7). He found that pyridine-3-sulfonic acid inhibited the growth of *Proteus* promoted by nicotinamide less strongly than it inhibited that promoted by diphosphopyridine nucleotide. On the basis of these results he postulates that nicotinamide must have some function other than acting as a building block for diphosphopyridine nucleotide, triphosphopyridine nucleotide, or both. He also obtained results with *Staphylococcus* which led to the same conclusion. Thus to the work reported in this paper there is added evidence of a different nature supporting the hypothesis that nicotinamide may act in respiration in some way other than as a building block for the nicotinamide-containing coenzymes.

SUMMARY

1. With glucose as substrate the respiration (or reduction of methylene blue) by dysentery bacilli grown on a medium deficient in nicotinamide can be stimulated not only by the addition of diphosphopyridine nucleotide or triphosphopyridine nucleotide but also by the addition of nicotinamide, nicotinic acid, methyl nicotinate, and other derivatives of nicotinic acid. The stimulation produced by the coenzymes is not as great as that produced by an equivalent amount of nicotinamide, but the activity of the coenzymes is increased by autoclaving and then becomes essentially equal to their nicotinamide content.

2. Methyl nicotinate is slightly more active than nicotinamide. Nicotinic acid is less active than the amide but becomes more active during the course of the experiment, indicating a possible synthesis of the amide from the acid.

3. When cells of *Proteus vulgaris* grown on a medium deficient in nicotinamide are used, their respiration with glucose as a substrate is stimulated by the same compounds which stimulate the respiration of the dysentery organism; but if lactate is substituted for glucose only intact diphosphopyridine nucleotide will stimulate respiration.

4. All the nicotinic acid derivatives which stimulate the oxidation of glucose by the dysentery organism also stimulate the oxidation of glucose monophosphate and glutamate. *Proteus* behaves towards glutamate as it does towards lactate but is unable to oxidize glucose monophosphate in the presence of nicotinamide or any of its derivatives.

5. The evidence presented is incompatible with the hypothesis that nicotinamide serves simply as a building block for diphosphopyridine nucleotide, triphosphopyridine nucleotide, or both, and the effects observed cannot be adequately explained by differences in rates of diffusion.

BIBLIOGRAPHY

1. Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Infect. Dis.*, **65**, 163 (1939).
2. Koser, S. A., and Saunders, F., *Bact. Rev.*, **2**, 99 (1938).
3. Fildes, P., *Brit. J. Exp. Path.*, **19**, 239 (1938).
4. Dorfman, A., Koser, S. A., Horwitt, M. K., Berkman, S., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, **43**, 163 (1940).
5. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 352 (1937).
6. Dorfman, A., Koser, S. A., and Saunders, F., *Science*, **90**, 544 (1939).
7. McIlwain, H., *Brit. J. Exp. Path.*, **21**, 136 (1940).

UTILIZATION OF GLYCOPROTEIN OF THE BLOOD PLASMA BY THE LACTATING MAMMARY GLAND*

BY E. P. REINEKE, M. B. WILLIAMSON, AND C. W. TURNER

(From the Department of Dairy Husbandry, University of Missouri, Columbia)

The recent investigations of Graham (1) and Shaw and Petersen (2) have demonstrated that when the rate of blood flow through the mammary gland is considered the uptake of amino acid nitrogen by the gland is insufficient to account for more than 25 to 30 per cent of the nitrogen in milk. Graham *et al.* (3) reported that the active mammary gland has the ability to synthesize urea and showed (4) that the output of urea nitrogen by this gland is approximately equal to the amount of amino acid nitrogen taken up from the blood passing through it. The above finding is supported further by the report of Shaw and Petersen (5) that active mammary gland tissue contains appreciable amounts of arginase. Further investigations on fractions of the non-protein nitrogen of the blood (6) indicate that uric acid, creatine, and creatinine are not of importance in the nitrogen metabolism of the lactating gland.

As a result of the nitrogen partition of arterial and mammary bloods, Graham, Peterson, Houchin, and Turner (4) reported evidence of a decrease in the level of globulin and an increase in the albumin fraction of the blood in passing through the lactating mammary gland. The theory was proposed that the milk proteins are not built up piece by piece from amino acids, but that part of the globulin molecule is used for the synthesis together with fractions of the non-protein nitrogen of the blood, and that the remainder of the globulin molecule is then returned to the venous blood as a portion of the albumin fraction.

Further investigations (7) have shown that the level of total nitrogen in both arterial and venous blood plasma in samples drawn from unanesthetized goats is subject to small variations from moment to moment, these variations being sufficient in some cases to mask arteriovenous differences of the magnitude that would be required to account for the nitrogen in milk. More recently it has been found[1] that goats will continue to secrete milk of normal composition at their normal rate under nembutal anesthesia, and that the effect of excitement of the goat during sampling on the level of various blood constituents is largely prevented by the use of animals previously anesthetized with nembutal.

The recent development of accurate quantitative methods for the determination of the carbohydrate portion of the plasma globulins appeared to offer a new approach to the difficult problem of characterizing the nitrogen fraction concerned in milk secretion.

<div align="center">EXPERIMENTAL</div>

Arterial and mammary venous blood samples were drawn from goats under nembutal anesthesia by the method of Graham, Turner, and Gomez (8). The blood was collected in flasks coated with equal amounts of potassium oxalate and sodium fluoride and chilled at once by immersion in ice water. Hemoglobin was determined by the method of Evelyn and Malloy (9). Amino acids were determined in a blood filtrate prepared according to Haden (10), by the method of Danielson (11). However, the actual colorimetric comparisons were made in an Evelyn photoelectric colorimeter, with Filter 520.[2] The initial galvanometer setting was made against a blank tube containing only the reagents used, and standard solutions for comparison were prepared for each set of determinations. For determination of the glycoprotein sugar the plasma proteins were precipitated and washed repeatedly with hot alcohol in a manner similar to that reported by Lustig and Ernst (12), except that in the final step the last traces of alcohol were removed by washing the precipitate with 3 ml. of ether. The precipitate was again centrifuged, the ether

---

[1] Reineke, E. P., Williamson, M. B., and Turner, C. W., to be published.

[2] This filter was selected on the basis of a careful spectrographic analysis made by V. R. Ells, Spectrographic Service, University of Missouri.

carefully decanted off, and the protein was dissolved in 0.5 cc. of 1 per cent NaOH. The carbohydrate content of the protein was determined by the orcinol-sulfuric acid method of Sörensen (13) as modified by Hewitt (14). The color was measured in an Evelyn photoelectric colorimeter, with Filter 490, and the sugar content was calculated against a standard of equal parts of mannose and galactose, which had been treated in the same manner as the dissolved protein. Correction for a small amount of color due to the action of sulfuric acid on the protein was made by use of a protein blank with the orcinol omitted. All analyses were run in triplicate, and the results were calculated from the two closest checks out of the three analyses.

A tabulation of the results obtained by this method in the experiments included in Series I showed an average difference between duplicates of 0.9 per cent. In later experiments, included in Series II and III, the margin of error was reduced to 0.5 per cent probably because of improved technique in manipulation of the protein precipitates.

The level of glycoprotein sugar, calculated as galactose-mannose, together with the arteriovenous differences in forty-two experiments is shown in Table I. For the arterial blood the values range from 86 to 158 mg. per cent, with an average of 105 mg. per cent.

The experiments with lactating goats (Series I) show an average uptake by the mammary gland of 2.15 mg. of glycoprotein sugar per hundred ml. of plasma, with a standard error of the difference of 0.395. Application of "Student's" (15) t test shows that there is less than one chance in a hundred that this difference could be due to chance variation.

The pregnant non-lactating goats used in this study were all in late stages of pregnancy when milk secretion processes are known to have begun, and the utilization of small amounts of milk precursors might be expected. Despite the fact that the arteriovenous difference of 0.58 mg. of sugar per ml. of plasma obtained for this series is but slightly larger than the average experimental error of the method, the statistical analysis indicates that the uptake of glycoprotein sugar is significant. No statistical significance can be attached, however, to the differences obtained for the non-pregnant, non-lactating goat.

TABLE I

Differences (in Mg. Per Cent) in Level of Glycoprotein Sugar in Arterial and Mammary Venous Plasma Samples from Lactating, Non-Lactating Pregnant, and Non-Lactating Non-Pregnant Goats

Goat No.	Series I. Lactating Glycoprotein sugar			Hb, per cent difference	Goat No.	Series II. Non-lactating pregnant Glycoprotein sugar			Hb, per cent difference
	Arterial	Venous	Difference			Arterial	Venous	Difference	
231	104.79	102.21	+2.58		507	92.60	93.06	−0.46	+0.34
231	99.74	100.89	−1.15	−0.65	444	90.22	88.84	+1.38	
231	87.44	84.93	+2.51	+0.98	842	107.98	107.01	+0.97	−0.64
231	89.47	88.03	+1.44	−0.49	842	103.37	103.26	+0.11	+1.17
231	100.17	96.54	+3.63	−0.60	842	121.50	122.49	−0.99	+0.24
231	126.60	123.87	+2.73	+0.96	842	120.23	119.07	+1.16	−2.66
135	97.64	100.15	−2.51	0	842	105.86	104.61	+1.25	+0.60
135	90.98	90.04	+0.94	+0.50	842	115.83	114.98	+0.85	+0.52
135	94.04	88.23	+5.81	+0.89	842	110.04	109.62	+0.42	−2.35
135	85.78	82.07	+3.71		842	128.29	127.82	+0.47	+0.06
842	99.10	97.01	+2.09	+0.17	842	126.67	125.68	+0.99	+0.67
842	85.82	85.82	0.00	+1.80	842	87.48	86.74	+0.74	−1.84
842	110.66	110.72	−0.06	+0.68	842	80.63	79.98	+0.65	−1.19
842	117.05	116.18	+0.87	−1.25					
842	117.27	113.27	+4.00	+0.86	Mean	106.97	106.39	+0.58	0.94
842	114.17	113.14	+1.03	+1.44					
842	157.59	152.83	+4.76	−0.15	Series III. Non-lactating non-pregnant				
842	110.62	108.40	+2.22	+1.52					
842	107.99	104.49	+3.50	+2.57					
842	98.68	97.52	+1.16	−1.50	439	90.67	90.01	+0.66	+0.48
842	113.58	111.83	+1.75		439	99.94	99.62	+0.32	−1.21
836	108.88	104.30	+4.58		439	95.46	94.74	+0.72	−0.84
836	87.01	83.56	+3.45	−1.13	439	105.29	105.41	−0.12	+1.87
438	116.49	114.03	+2.46	−0.64	439	96.99	96.68	+0.31	+0.53
Mean	105.07	102.92	+2.15	0.95	Mean	97.67	97.29	+0.38	0.98
Over-all mean for non-lactating goats ...						104.39	103.87	+0.52	0.95

Series No.	Mean arterio-venous difference	Standard error of difference	t	Degrees of freedom	Probability difference is due to chance
I	+2.15	0.395	5.440	23	Less than 1 in 100
II	+0.58	0.190	3.053	12	Approximately 1 in 100
III	+0.38	0.150	2.52	4	Significance not established
I vs. (II + III)		0.486	3.34	40	Less than 1 in 100

The authors are indebted to H. H. Kibler, Research Assistant, for the statistical computations.

If glycoprotein sugar is actually utilized by the mammary gland, one might expect that there would be a significantly higher uptake of this substance by the actively lactating compared to the inactive gland. Comparison of the arteriovenous difference for the lactating goats (Series I) with that for all non-lactating goats (Series II and III) by the pooled variance method (16) indicates that the uptake of this sugar by the lactating mammary gland is significantly higher.

The importance of considering the possibility of shifts in the water content of the blood in the interpretation of arteriovenous differences has been discussed by Shaw and Petersen (17). How-

TABLE II

Comparison of Level of Amino Acids in Arterial and Mammary Venous Blood from Lactating Fasted Goats

Goat No.	Amino acids			Duration of fast
	Arterial	Venous	Difference	
	mg. per cent	*mg. per cent*	*mg. per cent*	*hrs.*
836	10.02	10.16	−0.14	40
836	8.64	8.75	−0.11	64
836	10.89	11.31	−0.32	120
444	11.87	12.28	−0.41	48
444	8.44	8.32	+0.12	72
434	8.20	8.22	−0.02	42
434	8.82	8.96	−0.14	66
Mean	9.56	9.70	−0.14	

ever, by reference to the percentage hemoglobin differences between the arterial and venous bloods (Table I) it will be seen that the changes indicated are hardly significant, and that they show no relationship to the sugar differences. Another point to be considered is the fact that blood glucose is normally taken up by the lactating mammary gland, and if some free glucose were carried through with the protein it might account for the differences noted. However, in checking on the method used for isolating the proteins, it was found that the precipitated protein could be subjected to as many as six additional washings with hot 70 per cent alcohol or 80 per cent acetone with no appreciable change in the level of sugar or in the arteriovenous difference obtained.

During the course of investigations on lactating goats fasted for periods up to 120 hours, there was occasion to determine the amino acid content of arterial and mammary blood samples. Milk secretion continued in these goats, though at a reduced rate, but it will be noted by reference to Table II that there is no uptake of amino acid nitrogen by the lactating mammary gland in the fasting state. Unless the mammary gland has a greater storage capacity for nitrogenous milk precursors than hitherto suspected, this fact can be regarded as further evidence that the free amino acids of the blood are not essential for the formation of milk proteins.

DISCUSSION

The concept of a glycoprotein taking part in the nitrogen metabolism of the mammary gland is not entirely new. Hammond (18) on the basis of the constant ratio between the lactose and protein in milk suggested the possibility that the precursors of both substances are combined as a glycoprotein. Smith, Howat, and Ray (19) noted an unidentified constituent, which they suspected to be a glycoprotein intermediate, in certain samples of abnormal milk. However, the substance was not definitely identified.

The present report provides direct evidence that a small amount of the sugar that is conjugated with the proteins of the blood plasma is taken up by the lactating mammary gland. Because of the firm combination of the carbohydrate in the protein molecule, it may be assumed that the arteriovenous differences noted represent the uptake of glycoprotein or proteins as a unit. Since pure serum albumin contains no sugar (Hewitt (20), Kekwick (21)), it is apparent that the material absorbed must come from the globulin fraction, a conclusion that is in harmony with the report of Graham et al (4). Because of the fact that milk and particularly colostrum contain globulin that is serologically identical with that of the blood, there can be little doubt that the milk-secreting cells are permeable to globulin.

Although the arteriovenous differences obtained are believed to indicate definitely the uptake of a glycoprotein by the active mammary gland, no great quantitative significance can be attached to the absolute values because of the errors involved in small differences between two relatively large figures. However, by making some guided assumptions, it is possible to compute

roughly the nature of the substance involved. The rate of blood flow through the mammary gland as measured by the *Thermostrohmuhr* method is approximately 150 ml. of blood per ml. of milk produced (Graham (1)). To produce milk containing 500 mg. per cent of nitrogen an arteriovenous difference of about 3.3 mg. per cent of nitrogen would be required. If this is the case, the average uptake of 2.15 mg. per cent of mannose-galactose would indicate a glycoprotein containing about 10 per cent of sugar. Since the reports of Hewitt (22) and Bierry *et al.* (23) indicate that the blood globulins contain acetyl hexosamine in addition to the mannose-galactose measured by the orcinol reaction, this would be equivalent to 15 per cent of polysaccharides.

The highest carbohydrate content yet reported for a globulin fraction from normal blood is that of the "globoglycoid" of Hewitt, containing 9 per cent of galactose-mannose-glucosamine. However, Hewitt (22) reported that mild digestion of glycoprotein fractions with enzymes resulted in mucoid-like proteins with a high sugar content. It is not unlikely that the mammary gland contains enzymes that could activate a similar reaction. The complex with a high sugar content could then be retained, with the remainder being returned to the venous blood in a manner similar to that suggested by Graham, Peterson, Houchin, and Turner (4).

Sörensen and Haugaard (24) have reported that purified casein contains 0.31 per cent and lactalbumin 0.44 per cent of a sugar believed to be galactose. The derivation of milk proteins from glycoproteins of the blood plasma would obviously involve modification of these substances in the mammary gland, with some of the sugar probably being liberated for other requirements. Studies on the precursors of lactose (1, 2) indicate that the glucose plus lactic acid absorbed by the mammary gland will approximately account for the lactose in milk, but provides no excess for tissue metabolism or fat synthesis. Therefore, excess sugar derived from glycoprotein could be utilized to meet some of the carbohydrate requirements of lactation.

SUMMARY

By comparison of analyses of arterial and mammary venous blood samples drawn simultaneously from lactating goats it was shown that the mammary gland utilizes glycoproteins of the blood

plasma. The lactating gland showed an average uptake of 2.15 mg. of glycoprotein sugar per 100 ml. of plasma compared to 0.58 and 0.38 mg., respectively, for non-lactating pregnant and non-lactating non-pregnant goats. Since the glycoproteins of the blood are found only in the globulin fraction, this is considered to be further evidence that the plasma globulins are concerned in the synthesis of milk protein.

Arteriovenous differences for amino acid nitrogen, obtained on lactating fasted goats, showed no uptake of amino acids by the mammary gland, providing further evidence that free amino acids are not the major precursors of the protein in milk.

BIBLIOGRAPHY

1. Graham, W. R., Jr., *J. Biol. Chem.*, **122**, 1 (1937–38).
2. Shaw, J. C., and Petersen, W. E., *Am. J. Physiol.*, **123**, 183 (1938).
3. Graham, W. R., Jr., Houchin, O. B., and Turner, C. W., *J. Biol. Chem.*, **120**, 29 (1937).
4. Graham, W. R., Jr., Peterson, V. E., Houchin, O. B., and Turner, C. W., *J. Biol. Chem.*, **122**, 275 (1937–38).
5. Shaw, J. C., and Petersen, W. E., *Proc. Soc. Exp. Biol. and Med.*, **38**, 362 (1938).
6. Shaw, J. C., and Petersen, W. E., *Proc. Soc. Exp. Biol. and Med.*, **38**, 652 (1938).
7. Reineke, E. P., Peterson, V. E., Houchin, O. B., and Turner, C. W., *Missouri Agric. Exp. Stat., Research Bull. 296* (1939).
8. Graham, W. R., Jr., Turner, C. W., and Gomez, E. T., *Missouri Agric. Exp. Stat., Research Bull. 260* (1937).
9. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, **126**, 655 (1938).
10. Haden, R. L., *J. Biol. Chem.*, **56**, 469 (1923).
11. Danielson, I. S., *J. Biol. Chem.*, **101**, 505 (1933).
12. Lustig, B., and Ernst, T., *Biochem. Z.*, **289**, 365 (1937).
13. Sörensen, M., *Biochem. Z.*, **269**, 271 (1934).
14. Hewitt, L. F., *Biochem. J.*, **31**, 360 (1937).
15. "Student," *Metron*, **3**, 114 (1925).
16. Snedecor, G. W., Statistical methods, Ames (1937).
17. Shaw, J. C., and Petersen, W. E., *Proc. Soc. Exp. Biol. and Med.*, **42**, 520 (1939).
18. Hammond, J., *Quart. J. Exp. Physiol.*, **6**, 311 (1913).
19. Smith, J. A. B., Howat, G. R., and Ray, S. C., *J. Dairy Research*, **9**, 310 (1938).
20. Hewitt, L. F., *Biochem. J.*, **32**, 1554 (1938).
21. Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938).
22. Hewitt, L. F., *Biochem. J.*, **33**, 1497 (1939).
23. Bierry, H., Gouzon, B., and Magnan, C., *Compt. rend. Soc. biol.*, **130**, 411 (1939).
24. Sörensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933).

THE DETERMINATION OF THREONINE BY THE USE OF PERIODATE

By LEO A. SHINN and BEN H. NICOLET

(From the Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture, Washington)

Very shortly after the beginning of our work on the reaction of β-hydroxyamino acids with periodates, and before our first publication (1), Block and Bolling (2) announced a method for the determination of threonine in protein hydrolysates, in which the acetaldehyde formed by the action of lead tetraacetate was aerated into a solution of *p*-hydroxydiphenyl in sulfuric acid, and there determined colorimetrically. This method, as further developed (3), appears to be very useful, though most chemists, after reading the references (4, 5) they cite, would probably not join in their conclusion that "Eegriwe's method is highly specific for acetaldehyde."

In a paper in preparation, we shall discuss in detail the reaction of periodate on the naturally occurring amino acids in general. For the present, it is only necessary to report that serine and threonine react, under suitably chosen conditions, quantitatively and practically instantly and that the formaldehyde[1] and acetaldehyde thus formed may be separately determined with a very useful degree of accuracy.[2]

The acetaldehyde which is produced *almost immediately* from the reaction of periodate on threonine can be quantitatively car-

---

[1] Minimum values for serine of 3.6 per cent for casein and 1.9 per cent for gelatin will be reported. In the case of gelatin, there remains the question of how much of the formaldehyde was formed from hydroxylysine (6), which we have not had available for test.

[2] We wish to apply this determination of acetaldehyde in the presence of formaldehyde, in certain other directions, and specifically to the determination of methyl pentoses in the presence of pentoses and other carbohydrates.

ried over (without important contamination by formaldehyde[3]) by aeration at a pH close to 7, and preferably pH 7.0 to 7.2. If the aeration is carried out with CO_2, the maintenance of this range in a bicarbonate buffer is greatly facilitated.

We originally considered a method involving the aeration of acetaldehyde into a well cooled solution of dimedon. The pH varied during these experiments, and the precipitated product was less than the theoretical amount by a margin greater than the

TABLE I

Recovery of Threonine in Synthetic Mixtures*

Sample		Threonine recovered	pH at end of aeration
	mg.	*per cent*	
Threonine.	20	97.0	7.2
"	20		
Serine..	10	97.5	7.0
Alanine . . .	180		
Threonine	20		
Methionine	10	96 0	7.1
Alanine	180		
Threonine	20		
Serine	20		
Methionine	18	97.0	7 1
Cystine.	13		
Alanine	150		
Threonine...	5		
Tryptophane	10	98.3	7.1
Tyrosine.	10		

* The amino acids here tested for interference include all those which we have found to react importantly with periodate under these conditions.

solubility correction suggested by Vorländer (7). But the product obtained was not a color; it was a definitely characterizable organic compound. In our experience, the melting point determinations and mixed melting points showed it to be approximately pure.

[3] This is true of protein hydrolysates. In the case of relatively pure serine or threonine, the tendency for inconvenient amounts of formaldehyde to volatilize may be sufficiently restrained by the addition of a sufficient excess (10 molecules or more) of a suitable primary amine (we have chosen alanine).

We then shifted our interest to the bisulfite method. This permitted a much simpler and more quantitative recovery of acetaldehyde, together with a more rapid determination. The evidence of *specificity* in any given determination might, however, be less good. Conditions have, accordingly, been found under which this bisulfite solution may be treated to yield again its acetaldehyde for precipitation by dimedon. The product obtained is, in our experience, an approximately pure dimedon derivative. This secondary recovery has not been thoroughly investigated, and recoveries (without solubility corrections) of 80 per cent or more are considered satisfactory.

Our method in its routine form involves absorption of the selectively vaporized acetaldehyde in sodium bisulfite solution, with subsequent titration. The data in Tables I and II are offered as indications of its probable precision. In comparison with the method of Block and Bolling (2, 3), it offers both advantages and disadvantages.

We have preferred to use samples large enough to contain 5 mg. or more of threonine.[4] They are able to use considerably smaller quantities. We, on the other hand, offer the following advantages: (1) no special absorption tubes, (2) no colorimeter or screen, (3) no special purification of reagents, (4) no necessity for removal of chloride ion, (5) no reaction with, or correction for, alanine, (6) a possible check, on 5 mg. of threonine or less, of the purity of the aldehyde absorbed. (The dimedon derivative is identified by melting point and mixed melting point.)

It is felt that Table I adequately covers the question of interference by other commonly occurring amino acids. With regard to carbohydrates, we think the run with glucose (Table II) sufficient, since only methyl pentoses, which are not common contaminants of proteins, would be expected to behave differently.

With regard to the analyses of protein hydrolysates, it is apparent that our results for casein are in excellent agreement with those of Block and Bolling. In the case of gelatin, however, the disagreement is serious. Our confidence in our own results is

---

[4] We believe the work of Clausen (8) and of Troy and Sharp (9) is quite adequate evidence that the necessary sample can probably be reduced to one-tenth of this. We are quite content to leave this development to others who may be interested.

supported by the fact that we have *isolated* the equivalent of 1.18 per cent of threonine as the pure dimedon derivative of acetaldehyde.

For the present, we offer no explanation for this disagreement.

TABLE II

Determination of Threonine in Casein and Gelatin Hydrolysates, with Various Additions

Sample		Additions	HIO₄ (0.480 M)	Threo-nine found	Threo-nine found	Added threo-nine recov-ered
			cc.	*mg.*	*per cent*	*per cent*
0.25 gm. casein*			1.6	9.20	3.68	
0.25 " "		5 mg. threonine	1.6	14.06		97.0
0.25 " "			1.6	8.82	3.53	
0.25 " "			1.6	8.66	3.47	
0.25 " "			1.6	8.78	3.51	
0.25 " "			1.6	8.78	3.51	
0.50 " "			3.2	17.16‡	3.43	
0.25 " "			1.6	8.60‡	3.44	
0.25 " "			1.0	8.72	3.49	
0.25 " "		20 mg. glucose	2.0	8 67	3.46	
0.989 " gelatin†			3.0	14.80	1.49	
0.494 " "			1.5	7.23	1.47	
0.494 " "		5 mg. threonine	1.5	11.90		93.5
0.494 " "			1.5	7.05	1.43	
0.494 " "		{10 mg. serine 10 " hydroxyproline}	1.5	6.81	1.38	
0.500 " "			1.5	6.78	1.36	
0.500 " "		5 mg. threonine	1.5	11.62		97 0

* Weight refers to casein before hydrolysis. Sample was hydrolyzed 24 hours with 20 per cent HCl, and excess acid removed as usual by repeated evaporations *in vacuo*. There was no *chemical* removal of HCl beyond this.

† Weight refers to gelatin before hydrolysis. Sample was hydrolyzed 40 hours with 20 per cent HCl. For the rest, see note above.

‡ Aerated 2 hours.

EXPERIMENTAL

Apparatus—This consists essentially of three Pyrex test-tubes (2.5 × 20 cm.) fitted as a gas absorption train, except that Tube 1 carries a dropping funnel, the stem of which reaches nearly to the bottom of the tube which also serves in this case as the gas inlet tube. The acetaldehyde is produced in the first tube, and

absorbed in the second and third. It is useful to have a flow-meter to gage the rate at which CO_2 is passed during the determination.

Reagents—Those requiring reference are:

1. Sodium arsenite (0.1 N) containing 20 gm. of $NaHCO_3$ per liter.

2. Periodic acid (H_5IO_6),[5] approximately 0.5 M.

3. Sodium thiosulfate (0.1 N); the ultimate standard, to which various dilutions of this and of iodine are referred.

4. Dimethyldihydroresorcinol, 0.4 per cent aqueous solution.

5. Sodium bisulfite (2 per cent) containing 19 gm. of metabisulfite per liter.

Method

Before the reaction is started, the three tubes are charged as follows: Into the reaction Tube 1 are introduced in the indicated order (1) the sample,[6] preferably of a size to contain 3 to 10 mg. of threonine, and in a volume not much in excess of 5 cc., (2) 1 drop of Nujol, to reduce foaming, (3) 5 cc. of M sodium bicarbonate, (4) 10 cc. of sodium arsenite solution.

Tubes 2 and 3 should contain respectively 5 cc.[7] and 3 cc.[7] of 2 per cent sodium bisulfite, diluted in each case to 25 cc.

The apparatus is then connected to a source of CO_2, and gas passed for several seconds to mix the contents of all the tubes. The train is then broken to introduce into the funnel (stop-cock closed) 1 to 2 cc.[8] of 0.5 N periodic acid.

The system is reconnected, the stop-cock of the funnel opened, and the periodic acid allowed to flow in under CO_2 pressure.[9]

[5] G. Frederick Smith Chemical Company, Columbus, Ohio.

[6] If, instead of a protein hydrolysate, this is relatively pure threonine and also contains serine or carbohydrate, a quantity of alanine amounting to at least 10 molecules should be added here.

[7] Neither the volume nor the concentration need be *precisely* known.

[8] This depends on the size and nature of the sample. So long as the amount is between, say, 2 and 6 times that required for immediate reaction, the amount and concentration are not important.

[9] The use of this procedure guards against loss of acetaldehyde and also facilitates introduction of the reagent against back pressure from the following tubes. (These determinations can, of course, be run in series.) It also guards against contamination by acetaldehyde or acetone in the laboratory atmosphere.

Carbon dioxide is passed for 1 hour at the rate of about 1 liter per minute.[10]

At the end of this period, the contents of Tubes 2 and 3 are mixed and titrated according to the established procedure (8, 9) for acetaldehyde formed in the determination of lactic acid, except for the fact that we have used 0.02 N iodine where the authors cited used 0.002 N solutions. 1 cc. of 0.02 N iodine solution is equivalent to 1.19 mg. of threonine.

SUMMARY

A convenient method for the determination of threonine in protein hydrolysates has been described, based on the action of periodic acid. We consider this method superior, in many respects, to that described by Block and Bolling. Conditions have been developed for the selective removal of acetaldehyde, which is absorbed in bisulfite. Other applications of this procedure are being made.

Values for threonine of approximately 3.5 per cent for casein and 1.4 per cent for gelatin are reported.

BIBLIOGRAPHY

1. Nicolet, B. H., and Shinn, L. A., J. Am. Chem. Soc., 61, 1615 (1939).
2. Block, R. J., and Bolling, D., Proc. Soc. Exp. Biol. and Med., 40, 710 (1939).
3. Block, R. J., and Bolling, D., J. Biol. Chem., 130, 365 (1939).
4. Ecgriwe, E. S., Z. anal. Chem., 95, 323 (1933).
5. Miller, B. F., and Muntz, J. A., J. Biol. Chem., 126, 413 (1938).
6. Van Slyke, D. D., Hiller, A., MacFadyen, D. A., Hastings, A. B., and Klemperer, F. W., J. Biol. Chem., 133, 287 (1940).
7. Vorlander, D., Z. anal. Chem., 77, 327 (1929).
8. Clausen, S. W., J. Biol. Chem., 52, 263 (1922).
9. Troy, H. C., and Sharp, P. F., Cornell Univ. Agric. Exp. Stat., Mem., 179 (1935).

---

[10] This time is usually sufficient. It is, of course, very easy to test any suspicion of insufficiency.

STUDIES ON HUMAN SERUM PROTEINS

II. CRYSTALLIZATION OF HUMAN SERUM ALBUMIN

By FORREST E. KENDALL

(*From the Research Service, First Division, Welfare Hospital, Department of Hospitals, and the College of Physicians and Surgeons, Columbia University, New York*)

Although the crystallization of albumin from human serum has been reported by Adair and Taylor (1), crystalline preparations are not consistently yielded by the methods they describe. The procedure described in the present report has given good yields of crystalline albumin in every case when applied to twenty-one samples of normal human sera. The sera were derived from healthy adults or from citrated blood discarded from a blood bank because it was overage or because the donor had an infection that rendered the blood unsuitable for transfusion. Some of the samples of blood bank plasma had been stored for as long as 6 months before fractionation, many of the samples were badly hemolyzed, and some of them were highly lipemic. In some cases the citrated plasma was fractionated directly, and in others it was first defibrinated by clotting with $CaCl_2$ or inactivated by heating to 56° for 30 minutes. None of these factors influenced the yield of crystalline albumin.

Isolation of Crystalline Human Serum Albumin

100 ml. of undiluted serum or plasma were chilled in an ice bath[1] and 120 ml. of ammonium sulfate solution, saturated at room temperature, were slowly added from a dropping funnel, over the period of an hour, with mechanical stirring. After vigorous stirring for another hour the precipitated globulin was

---

[1] While temperature control at this point was not essential for the isolation of the crystalline albumin, it was needed to prevent changes in the globulin fractions.

filtered off in the cold with suction.   The filtration was repeated
if necessary to give a clear filtrate.   The albumin was then pre-
cipitated by dissolving 25 gm. of solid ammonium sulfate in the
filtrate (75 per cent saturation)   After standing overnight at
room temperature the amorphous precipitated albumin was
filtered off with suction.   The moist precipitate was dissolved in
25 ml. of distilled water and the volume of the solution measured.
The hydrogen ion concentration was adjusted to pH 4.9[2] with
0.1 N H_2SO_4 in half saturated ammonium sulfate.   The solution
was cooled to 15° and the ammonium sulfate concentration
brought to half saturation by the addition of the calculated
amount of saturated solution.   The amount needed was 39 ml.
minus one-half the measured volume of the albumin solution.
This formula assumes the presence of 4 gm. of albumin and makes

Fig. 1. Crystalline human albumin × 75

allowance for the 75 per cent saturated ammonium sulfate solution
in the moist precipitate.   A small precipitate formed which was
filtered off through a folded filter in the ice box.   The clear
filtrate was allowed to stand in the ice box at 1° until crystalliza-
tion occurred.   Crystallization was very slow, requiring in some
cases a period of 2 weeks for the appearance of the first crystals.
Although seeding the solution with crystals accelerated the
crystallization, at least 2 weeks more were necessary for maximum
yields.   With the exception of a small amount of flocculent amor-
phous material which sometimes separated before crystallization
began, the precipitate was entirely crystalline, consisting of
elongated duodecahedrons with square cross-section (Fig. 1),
which exhibited the property of double refraction of polarized
light (Fig 2).   The crystals were very fragile, being broken up by

[2] All hydrogen ion concentrations were determined electrometrically
with a glass electrode.

vigorous stirring or by the pressure of a cover-glass on a micro-scope slide. They showed a high temperature coefficient of solubility, going into solution in the supernatant fluid if warmed to room temperature, when they were often replaced by amorphous material. From 40 to 65 per cent of the albumin was obtained in crystalline form. The yield could be increased and a second crop of crystals obtained either by concentrating the supernatant solution by ultrafiltration at 0° or by precipitating the remaining albumin at 75 per cent saturation with ammonium sulfate and repeating the crystallization procedure from a smaller volume.

FIG. 2 Crystalline human albumin × 75. Polarized light

Recrystallization of Human Serum Albumin

The crystals from 100 ml. of serum were dissolved in 10 ml. of water and filtered through an asbestos filter. 10 ml. of saturated ammonium sulfate solution were added at 15° without causing precipitation. The solution was cooled to 4° and seeded with crystals. Although crystallization was more rapid than before, at least a week was required to give maximum yields. After four crystallizations the crystals were dissolved in a small volume of H_2O and dialyzed in the cold against distilled water until free from ammonium sulfate. The pH of the dialyzed solution was 4.9.

Several preparations were dried *in vacuo* while frozen. The dried material dissolved completely in water to give clear solu-

tions that crystallized when half saturated with ammonium sulfate. Samples of different preparations when dried to constant weight *in vacuo* over P_2O_5 at room temperature had a nitrogen content of 15.7 $\pm$ 0.2 per cent by the micro-Kjeldahl method. All preparations were free from carbohydrate when analyzed by the orcinol method of Sørensen and Haugaard (2).

Fractionation of Crystalline Human Albumin

The albumin which had been crystallized four times was still colored yellow by the presence of serum pigments. However, the first crystals to separate were more highly colored than later fractions. Therefore an attempt was made to obtain colorless preparations by fractional crystallization.

8 gm. of albumin which had been crystallized four times from half saturated ammonium sulfate were dissolved in 50 ml. of H_2O, chilled to 10°, and treated with 30 ml. of saturated ammonium sulfate. The clear solution was seeded with crystals and allowed to stand at 0°. Crystallization proceeded rapidly, forming crystals indistinguishable in form from those obtained from *half* saturated ammonium sulfate. After 3 days the crystals were centrifuged down compactly in the cold and redissolved in 25 ml. of water. Upon the addition of 15 ml. of saturated ammonium sulfate solution crystallization took place as before. After two more recrystallizations the crystals were dissolved in a small volume of water and dialyzed against distilled water in the cold until free from ammonium sulfate. The pH of the dialyzed solution was 4.9. The yield of Fraction I was 4.2 gm.

The supernatant solutions from these crystallizations were mixed and ammonium sulfate added to half saturation. Crystallization took place slowly after the solution had been seeded with crystals, 3 weeks at 0° being required to obtain a maximum yield. After three more crystallizations from half saturated ammonium sulfate this fraction was dialyzed free from salts. The yield of Fraction II was 1 3 gm. Fraction I was still highly colored with the yellow pigment, while Fraction II was almost colorless.

Lipid Content of Crystalline Human Albumin

It was suspected that this pigment might be lipoidal in nature. Therefore a dried sample of albumin crystallized four times was

extracted with dry ether for 24 hours in a Soxhlet extractor. 0.48 gm. of albumin gave 0.0026 gm. of ether-soluble material, 0.5 per cent lipid. The ether-extracted albumin retained most of the original pigment. After removal of the ether *in vacuo* the albumin was still completely water-soluble and crystallized upon the addition of ammonium sulfate.

However, if the albumin was denatured by heating in aqueous solution and extracted with hot alcohol, larger quantities of lipid were obtained which contained the yellow pigment. The alcoholic extract was evaporated to dryness and the lipid was extracted from the residue with dry chloroform. Table I shows that four preparations of albumin crystallized four times contained between 1.8 and 2.9 per cent of lipid and that Fractions I and II, obtained

TABLE I

Lipid Content of Crystalline Human Albumin

Preparation No	Sample	Lipid	Lipid	Acid equivalent
	gm.	*gm.*	*per cent*	
1	0 998	0 0220	2.2	280
2	0.291	0 0084	2 9	318
3	0.175	0 0039	2 2	
3 (Fraction I)	0.252	0 0033	1 3	
3 (" II)	0 169	0.0007	0 4	
4	0 210	0.0038	1 8	

by fractional crystallization, contained 1 3 and 0.4 per cent respectively. The lipid was completely soluble in acetone and was free from cholesterol. Two samples when titrated in alcoholic solution with NaOH with phenolphthalein as indicator gave acid equivalents of 280 and 318   The lipid could be extracted from chloroform or ether with dilute NaOH and reextracted from the aqueous solution with ether after acidification. Both the color and the odor associated with human serum followed the lipid through these extractions   It would thus appear that crystalline human serum albumin is associated with a free fatty acid which cannot be extracted without first denaturing the albumin. The amount of this lipid varies in different preparations and the albumin can be separated into fractions having different contents of

fatty acid.   The nature of this fatty acid is to be investigated when sufficient quantities for study have been accumulated.

Homogeneity of Human Crystalline Albumin

The isolation of a protein in crystalline form does not insure the homogeneity of the preparation.   Mixed crystals may be formed which contain more than one protein as in the case of the crystalline chymotrypsins (3) or a non-protein constituent as in the case of crystalline pepsin (4).   Impurities may be adsorbed by the crystal surfaces and carried through many recrystallizations.

Fig 3 Electrophoresis diagram of crystalline human albumin after 3 hours. Left, descending boundary, right, ascending boundary

Some information concerning the homogeneity of a protein preparation can be obtained by electrophoresis.   A sample of Preparation 3 of the albumin crystallized four times was analyzed electrophoretically in the Tiselius apparatus[3] at pH 7.34 (0.02 M phosphate buffer and 0.15 M NaCl) and at an albumin concentration of 1.1 per cent.   It was found to be homogeneous, as indicated by the Longsworth pattern (5).   The photograph in Fig. 3 was taken after 3 hours of migration in a field of 2.62 volts per cm., giving a mobility of $5 33 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}.

[3] The measurements were made by Dr Dan H Moore of the Electrophoresis Laboratory, College of Physicians and Surgeons, Columbia University.

The albumin mobilities of different samples of normal human plasma under similar conditions were found to be 4.9, 4.5, 4.6, 4.8, and 5.7 $\times$ 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

The sharpness of the diagram obtained with crystalline human albumin cannot be taken as definite proof that only one protein is present. Although the albumin in plasma migrates as a single boundary, Hewitt (6) has shown that it can be fractionated into a carbohydrate-containing albumin, "seroglycoid," and a carbohydrate-free "crystalbumin."

The most sensitive criterion of the homogeneity of a protein preparation is furnished by solubility measurements. The solubility of a pure protein should be independent of the amount of solid protein in equilibrium with the saturated solution (7). Successive equilibrations of the solid protein with fresh solvent should yield constant values (8).

The solubility of the crystalline human serum albumin and the two samples obtained by fractionation with ammonium sulfate was studied in a number of ways. The solubility in 2.0 M (NH$_4$)$_2$-SO$_4$ solution at pH 4.9 and 0° was determined by approaching the final equilibrium in two ways: first, by allowing the albumin to crystallize from supersaturated solutions and, second, by equilibrating an excess of the crystals with 2 M (NH$_4$)$_2$SO$_4$.

10 ml. samples of solution containing varying amounts of albumin were mixed with 10 ml. of 4.0 M (NH$_4$)$_2$SO$_4$ at 15°. The clear solutions were cooled to 0° in an ice bath and seeded with a . few crystals. The solutions were stirred for 2 hours and then allowed to stand for 24 hours at 0°. At that time the crystals were centrifuged down compactly at 0° and duplicate 1 ml. samples of the clear supernatant solutions were taken for analysis. The crystals were then resuspended in the remaining solution by stirring for 1 hour and kept in an ice bath at 0°. Similar samples for analysis were taken at intervals of several days until constant values were obtained. The pH and the ammonium sulfate concentration were determined at the end of each experiment. Microscopic examination showed that in each case the final precipitate was entirely crystalline and free from amorphous material. The samples for analysis were diluted to 10 ml. with H$_2$O and heated on a boiling water bath for 10 minutes. The coagulated protein was filtered through a porous porcelain crucible and

washed free from $(NH_4)_2SO_4$ with hot water.   The albumin was then dissolved in 0.1 N NaOH and washed into a Kjeldahl flask. Nitrogen was determined by the micro-Kjeldahl method.

Table II shows that at least 3 weeks were required to reach constant values.   In four of five experiments the final values were

TABLE II

Solubility of Crystalline Human Serum Albumin in
2.0 M $(NH_4)_2SO_4$ at 0°, pH 4.9

	Equilibration of crystals with supersaturated albumin solution									
	Preparation 1				Preparation 3					
Lipid content, per cent .. .	2.2				Unfractionated 2.2		Fraction I 1 3		Fraction II 0.4	
Albumin in original solution, *gm*	0 210*		0.420*		0.566*		0 346*		0.346*	
	Time	Solu-bility	Time	Solu-bility	Time	Solu-bility	Time	Solu-bility	Time	Solu-bility
	days	*mg. per ml.*	*days*	*mg. per ml.*	*days*	*mg. per ml.*	*days*	*mg. per ml.*	*days*	*mg per ml.*
	1	9.7	1	13.0	2	14 9	5	7.8	5	16.0
	3	9.5	3	10.8	9	9.0	11	5.8	11	14.3
	5	9.2	5	10.3	16	7.8	16	5.2†	16	11.6†
	8	8.8	8	9.6	22	7.4	19	4.8†	19	10.6†
	11	8.4	11	8.9	28	7.4	25	4.7†	25	9.5†
	16	7.9	16	7.8			32	5.0†	32	8.8†
	23	7.6	23	7.7			39	5.4†	39	8.1†
							48	4.8	48	8.1
Final concentration, $(NH_4)_2SO_4$, M...	2.04		2.04		2.05		2.03		2.03	
Final pH. . ..	4.9		4.9		4.9		4.88		4.92	
Albumin in final ppt. (calculated)	0.042 gm.		0.238 gm.		0.401 gm.		0.237 gm.		0.144 mg.	

* Albumin values = nitrogen ÷ 0.157.
† Single determinations.

the same, within experimental variation, and were independent of the amount of crystalline protein present.   In the fifth experiment, in which the solubility of Fraction I which had been crystallized from 1.5 M $(NH_4)_2SO_4$ was measured, the solubility was much lower.

In three cases in which an excess of crystals was equilibrated with fresh solvent, Preparation 3 and Fractions I and II, the crystals used were those obtained in the previous experiment in which they had been equilibrated with a supersaturated solution. In

TABLE III

Solubility of Crystalline Human Serum Albumin at 0°, pH 4.9

Albumin, initial, *gm*	Equilibration of crystals with 20 ml. 2 0 M (NH₄)₂SO₄							
	Preparation 1		Preparation 3					
	2 0		Unfractionated 0.401*		Fraction I 0.237*		Fraction II 0.144*	
	Time	Solubility	Time	Solubility	Time	Solubility	Time	Solubility
	days	*mg. per ml.†*	*days*	*mg. per ml.†*	*days*	*mg. per ml.†*	*days*	*mg. per ml.†*
	1	4.2	2	3.7	6	1.5	6	1.4
	2	5.0	9	3.9	12	1.4	12	1.4
	3	5.3	27	3.9				
	4	5.3						
	7	5.3						
Final concentration, (NH₄)₂SO₄, *M*	2.10		2.04		2.03		2 03	
Final pH	4.9		4 9		4.9		4.9	

Equilibration of crystals with successive 20 ml. samples of 2 02 M (NH₄)₂SO₄

Preparation 4, 1 8 per cent lipid, 1.8 gm.

1st equilibration		2nd equilibration		3rd equilibration		4th equilibration	
days	*mg. per ml.†*	*days*	*mg. per ml.†*	*days*	*mg. per ml.†*	*days*	*mg. per ml.†*
2	2.2	2	2.2	2			
4	2.8	4	2.7	4	2.6		
7	2.9	7	2 9	7	2.9	7	2.9

* Crystals from previous equilibration with supersaturated albumin solution.

† Albumin values = nitrogen ÷ 0.157.

two cases, Preparations 1 and 4, the crystals were freshly prepared from the dialyzed stock solutions. Table III shows that constant values for solubility were reached in about 3 days and that these values were markedly lower than those found when the crystals were equilibrated with the supersaturated solution and were

different for different preparations. Four successive equilibrations of fresh solvent with the crystals from Preparation 4 gave the same solubility for each equilibration.

It seems evident that in these experiments a true equilibrium was never established between the crystals and the solution, although constant solubility values were obtained. The presence of the fatty acid in the crystals might account for this. If the crystals are built up of albumin and fatty acid molecules, the apparent solubility would depend upon the nature of the surface of the crystal in contact with the solution and the nature of the surface would depend upon the manner in which it is formed. If the crystal is produced by the deposition of layers of albumin and fatty acid from a supersaturated solution, the crystal would continue to grow until an equilibrium was established between the surface and the more soluble component in solution. If the crystal is equilibrated with fresh solvent, it would tend to dissolve until a layer is formed which is in equilibrium with the more insoluble component in the solution. In one case the surface would be largely albumin and in the other largely fatty acid. This would not represent a true equilibrium but under the conditions of the experiment the surfaces may be stable enough to prevent the establishment of a true equilibrium within periods of time that are practical experimentally.

This surface effect would not be present if the protein were precipitated in an amorphous state where there is no orientation of the albumin and fatty acids. Therefore the solubility of the amorphous albumin precipitated by sodium sulfate was studied. Different sized samples of albumin crystallized four times and of Fractions I and II were made up to 25 ml. with sodium sulfate and phosphate buffer of pH 6.8. After incubation for 18 hours at 37° the precipitated albumin was filtered off and duplicate 10 ml. samples of each filtrate analyzed for nitrogen by the micro-Kjeldahl method. The phosphate concentration was 0.12 M in each case and the sodium sulfate concentration varied between 2.05 M and 2.26 M. The results given in Table IV show that the solubility of the albumin is independent of the amount of solid in contact with the saturated solutions. The great difference found between Fraction I and the other preparations when the solubility of the crystalline albumin was measured does not appear. How-

ever, somewhat anomalous results were obtained with Fraction I in this experiment in that the solubility apparently decreased as the amount of protein increased. This may be due to a stabilizing effect of the fatty acid present which might prevent the coagulation of small amounts of precipitated protein. However, this effect was not observed in the case of the unfractionated albumin which contained a larger amount of fat.

While these solubility measurements do not show unequivocally that crystalline human serum albumin contains but a single protein component, they do indicate that the albumin is much more homogeneous than the corresponding albumin prepared from horse serum. The solubility of crystalline horse serum albumin is dependent upon the amount of solid in equilibrium with the solution (9). That this behavior is due to the inhomogeneity of the albumin is confirmed by Ferry (10) and McMeekin (11) by the isolation of part of the albumin as a crystalline sulfate which shows constant solubility. Attempts to prepare a similar sulfate from human serum albumin have not been successful.

It is suspected that these differences may not be due to a greater complexity of the albumin as it exists in the blood of a horse, but that they may result from changes occurring during the isolation and fractionation of the crystalline albumin. The procedures which have been used for the isolation of crystalline horse albumin are more drastic than those described in this paper for the preparation of crystalline human albumin. Application of the more drastic methods to human serum leads to amorphous preparations. However, if saturated solutions of the crystalline human albumin in 2.0 M $(NH_4)_2SO_4$ at pH 4.9 are allowed to stand in open vessels at room temperature so that slow concentration of the solution occurs, a new type of crystalline albumin separates out. These crystals are extremely small needles, often forming sheaves or bundles similar to those formed by egg albumin. After the albumin is once obtained in this form, it may be recrystallized by dissolving it in a small amount of water and adding saturated $(NH_4)_2SO_4$ until a permanent turbidity is formed and then allowing it to stand at room temperature. A preparation recrystallized four times at room temperature had a lipid content of 2.3 per cent as compared with 2.2 per cent for the starting material. Solubility measurements were made upon a sample obtained from

Preparation 3 and recrystallized four times at room temperature. The results given in Table IV show that this preparation now behaves like horse albumin in that its solubility increases as the amount of solid in contact with the solution is increased. Since the albumin from which this preparation was made showed con-

TABLE IV

Effect of Size of Sample upon Solubility of Human Serum Albumin in 25 Ml. of Na₂SO₄ Solution

Buffered with 0.12 M phosphate at pH 6.8, $t = 37°$.

Salt concentration	Preparation 3					
	Albumin, recrystallized 4 times, 2.3 per cent lipid		Fraction I, 1.3 per cent lipid		Fraction II, 0 4 per cent lipid	
	Total albumin N	Filtrate N*	Total albumin N	Filtrate N	Total albumin N	Filtrate N
moles per liter	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg*
2.17	3.10	No ppt.	2.16	No ppt.	2.16	No ppt
2.17	6.21	3.06	4.32	" "	4.32	3.13
2.17	9.32	3.05	6.48	4.30	6.48	3.32
2.17			8.64	3.63	8.64	3.30
2.17			13.50	3.88	13.50	3.33
2.17			20.25	3.40	20.25	3.38
2.24	3.10	0.62				
2.24	6.21	0.63				
2.31	3.10	0.20				
2.31	6.21	0.19				
2.38	3.10	0.01				
	6.21	0.01				
Crystals isolated at room temperature; Preparation 3a, 2.3% lipid						
2.24	2.30	0.80				
2.24	4.60	0.97				
2.24	6.90	1.15				

* The values for nitrogen in the filtrate were calculated upon the assumption that the volume of the filtrate was 25 ml. No correction was made for the volume of the precipitate.

stant solubility (Table IV), the inhomogeneity of this preparation must be due to changes taking place during its isolation.

SUMMARY

1. A method is given for the isolation of crystalline albumin from human serum.

2. The crystalline albumin is associated with a small amount of free fatty acid that cannot be removed without first denaturing the albumin.

3. Solubility measurements indicate that the crystalline human serum albumin is more homogeneous than the corresponding albumin isolated from horse serum.

BIBLIOGRAPHY

1. Adair, M. E., and Taylor, G. L., *Nature*, **135**, 307 (1935).
2. Sørensen, M., and Haugaard, G., *Compt.-rend. trav. Lab. Carlsberg*, **19**, No. 12 (1933).
3. Kunitz, J., and Northrop, J. H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, New York, **6**, 325 (1938).
4. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, **13**, 781 (1930).
5. Longsworth, L. G., *J. Am. Chem. Soc.*, **61**, 529 (1939).
6. Hewitt, L. F., *Biochem. J.*, **30**, 2229 (1936).
7. Sørensen, S. P. L., and Høyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, **12**, 213 (1917). Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, **6**, 131 (1923). Sørensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **15**, No. 11 (1925).
8. Steinhardt, J., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, New York, **6**, 301 (1938).
9. Sørensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **18**, No. 5 (1930).
10. Ferry, J. D., reported to the Division of Biological Chemistry, American Chemical Society, 96th Meeting, Milwaukee, September 7 (1938).
11. McMeekin, T. L., *J. Am. Chem. Soc.*, **61**, 2884 (1939).

ASCORBIC ACID DEFICIENCY AND ENZYME ACTIVITY IN GUINEA PIG TISSUES*

By CARTER J. HARRER and C. G. KING

(From the Department of Chemistry, University of Pittsburgh, Pittsburgh)

Independently of, or parallel with, the possible function of ascorbic acid as a carrier-catalyst in tissue respiration the vitamin may be of major importance in cellular physiology as a regulating and protective agent. The present investigation is concerned with the effects of ascorbic acid deficiency upon the activity of two hydrolytic and two respiratory types of enzymes in guinea pig tissues: phosphatase of intestinal mucosa, liver, kidney, and brain; liver esterase; succinic dehydrogenase of skeletal and heart muscle; and cytochrome oxidase of skeletal muscle, heart, and brain.

Hellerman (1) has reviewed the literature on the reversible inactivation of certain hydrolytic enzymes. In many cases it is apparent that oxidative reversibility conforms to a postulated sulfhydryl group behavior of the type $2R\text{---}SH \rightleftharpoons R\text{--}SS\text{---}R + 2(H)$, in which only one form of the enzyme molecule is active and in which R— represents a protein, either directly or through linkage with a coenzyme group. By protecting such active groups, either directly or indirectly, relatively small amounts of ascorbic acid might aid in the regulation of major hydrolytic and respiratory systems in the tissues without entering directly into the reactions concerned. From the studies of reactions with ascorbic acid of the quinone and quinonimide type in our own and in other laboratories it appears very probable that ascorbic acid functions in part at least through such agencies (2).

Kraut and Pantschenko-Jurewicz (3, 4) have suggested that liver esterase is composed of a protein fraction (apoenzyme) linked with an active ascorbic acid group (coenzyme). According to these authors ascorbic acid catalyzed the hydrolysis of simple esters in the presence of totally inactive protein which had been separated from liver esterase. Kertesz (5) and Strachitskiĭ and Meerzon (6) were unable to confirm their findings. Earlier studies of liver esterase in our laboratory, particularly those showing the stability of the enzyme against traces of copper, oxygen, and other oxidizing agents, made it unlikely that the vitamin constituted an active part of the enzyme. Scoz, Cattaneo, and Gabrielli (7) and Raabe (8) observed a decrease in the liver esterase of vitamin C-deficient guinea pigs, and Raabe noted a rise in esterase activity when the vitamin was supplied, but he doubted the coenzymic rôle of ascorbic acid and suggested a protective action instead.

Reports concerning the relation of vitamin C to phosphatase activity have been very conflicting. Giri (9) reported that oxidized ascorbic acid, prepared by means of cupric ion catalysis or by direct oxidation with ferricyanide or methylene blue, inhibited plant phosphatase. Activity was restored by treatment with —SH compounds and other reducing agents. The same author later reported that dialyzed phosphatase from animal tissue was inhibited by ascorbate-Cu^{++} (10). Von Euler and Svenson (11) observed that ascorbic acid caused a slight inhibition of the phosphatase activity of hog intestinal mucosa. Clinical studies have indicated that plasma phosphatase activity remains nearly normal during acute scurvy, but rises during curative treatment coincident with increased calcification (12). Serum phosphatase was reported to drop below normal during chronic scurvy, parallel with retarded growth (13). Direct activation of serum phosphatase by ascorbic acid apparently does not occur to a significant degree (14, 15).

Of the respiratory enzymes studied by Hopkins and his associates (16, 17), only succinic dehydrogenase showed a marked correlation between activity and the apparent presence of sulfhydryl groups. Rapkine (18) and Rapkine and Trpinac (19) made similar observations. The triose phosphate-pyruvate dismutation is also in part dependent upon the integrity of the pro-

tein sulfhydryl groups. The findings of Ecker and associates regarding the dependence of complement activity upon sulfhydryl groups and their protection in turn by ascorbic acid (20) are of interest in relation to the above observations.

<div align="center">EXPERIMENTAL AND DISCUSSION</div>

Liver Esterase—Because of the suggested relation between liver esterase and ascorbic acid, we first compared the esterase activities of normal and scorbutic guinea pig liver. The activity of both fresh tissue suspensions and of extracts from acetone-dried liver was determined by the Knaffl-Lenz procedure (21). After 2.0 ml. of a saturated solution of redistilled ethyl butyrate, 0.25 ml. of 0.1 per cent brom-thymol blue, and enough water to give a final volume of 8.0 ml. were mixed, a suitable volume of enzyme in the form of tissue suspension or extract was added to the mixture. The pH was continuously adjusted by drop titration over a half hour period by the addition of standardized sodium hydroxide. Slight variations in room temperature had no noticeable effect on esterase activity measured in this way. The enzyme activity was expressed as ml. of 0.01 N alkali required to neutralize the acid produced per mg. of dry weight of tissue or enzyme preparation. The rate of hydrolysis of ethyl butyrate was proportional to the amount of tissue used and an approximately constant reaction rate could be maintained through short periods.

The esterase activity was determined in suspensions of fresh tissue from three groups of guinea pigs. Animals of the following types were used: (a) 500 gm. animals maintained on the Sherman basal ration (rolled oats, bran, butter fat, heated skim milk powder, salt, cod liver oil, and yeast), supplemented by 10 gm. or more of fresh, crisp spinach; (b) animals on the basal ration without spinach or other ascorbic acid supplement; and (c) assay animals that received 0.5 or 2.0 mg. of ascorbic acid daily for 21 days after a preliminary depletion period of 10 to 12 days. The water suspension of homogenized tissue contained approximately 35 mg. of fresh liver per ml.

The esterase activities of fresh tissues prepared in this manner are recorded in Table I, expressed as ml. of 0.01 N alkali consumed per 10 minutes per mg. of dry weight. It will be noted that the esterase activity of the assay animals, known to be low

in ascorbic acid content, and of the severely scorbutic animals, nearly devoid of ascorbic acid, was lower by approximately 25 and 65 per cent respectively than that of the normal animals.

TABLE I

Liver Esterase Activity in Normal and Vitamin C-Depleted Tissue

Homogenized fresh liver; ethyl butyrate substrate, 0.0146 M. The activity is expressed as ml. of 0.01 N alkali per mg. of dry tissue, per 10 minute interval.

Normal, esterase activity	Low vitamin level			Scorbutic	
	Vitamin supplement	Scurvy score	Esterase activity	Scurvy score	Esterase activity
	mg.				
0.65	0.5	4	0.63	14	0.37
0.62	0.5	1	0.84	17	0.36
0.80	0.5	3	0.43	16	0.47
0.92	0 5	5	0.71	19	0.28
0.82	0.5	4	0.83	17	0.21
1.17	0.5	5	0.64	16	0.21
1.17	2.0	4	1.16	19	0.12
1.20				15	0.65
1.36				17	0.33
0.74				19	0.18
				17	0.38
				20	0.37
				19	0.33
				10	0.30
				8	0.23
				8	0.43
				6	0.30
				10	0.28
				15	0.30
				12	0.26
				20	0.39
Average. 0.95 ±0.053			0.75 ±0.05		0 32 ±0.016

The esterase activity of partially purified extracts of acetone-dried liver from normal and scorbutic animals was also measured. Extracts of acetone-dried liver were made according to the procedure outlined in a previous publication (22). Extreme variation of esterase activity was found for different animals in the

same group. The observed difference between normal and scorbutic fresh tissue suspensions was not apparent after the purification procedure. The stability of the preparations, both fresh and dried, was far too great, however, to correspond with the properties one would expect if ascorbic acid were an active part of the enzyme. The results illustrate the marked protective action by ascorbic acid, however, in maintaining normal esterase activity in the fresh tissue (and presumably in the living organism).

Phosphatase—The method of Kay (23), with slight modifications, was used for the determination of phosphatase activity in normal and scorbutic guinea pig intestinal mucosa, kidney cortex, brain, and liver. The animals used in this and the following studies were very similar to those used in the measurement of esterase activity.

Portions of the tissues mentioned above were homogenized and allowed to stand in chloroform water for 24 hours at room temperature. The filtrate obtained after the suspension was passed through cotton was diluted to the desired concentration and used in the determination of phosphatase activity.

1 ml. of phosphatase preparation equal to about 50 mg. of intestinal mucosa, 25 mg. of kidney cortex, 100 mg. of brain, or 100 mg. of liver, dry weight, was added to 20 ml. tubes containing 5.0 ml. of a mixture of sodium β-glycerophosphate (0.2 M) and magnesium sulfate (0.003 M), 4.0 ml. of glycine and NaCl-NaOH buffer (pH 9.0), and water to a final volume of 10.0 ml. Two control tubes contained the above reagents without enzyme and without substrate respectively. After thorough mixing, the tubes were stoppered and incubated at 37.2° for periods of 2 to 6 hours. Aliquots (2.0 ml.) were removed at the proper time and pipetted into 8.0 ml. of 10 per cent trichloroacetic acid. After centrifuging, aliquots of the acid supernatant were analyzed for inorganic phosphate by the method of Fiske and Subbarow (24). The amounts of tissue used and the volumes of acid supernatant taken for phosphate analysis were adjusted to give a suitable colorimeter reading when compared with a standard containing 0.004 mg. of phosphorus per ml. In some cases standard phosphate was added to the unknown, and the amount of hydrolyzed phosphorus was calculated by difference. All values reported

were corrected for the amounts of inorganic phosphorus found in the controls accompanying each experiment. Individual experiments showed that the rate of hydrolysis was proportional to the amount of extract used and that the rate gradually fell off during the course of the experiments.

The results given in Table II show that there was a moderate but irregular decrease in the phosphatase activity of the intestinal mucosa and kidney cortex during severe scurvy; the decreases in brain and liver were not considered as significant. It is un-

TABLE II

Phosphatase Activity of Kidney Cortex, Brain, and Liver

T 37.2°; pH 9.0; time of hydrolysis, 6 hours. The activity is expressed as mg. of phosphorus hydrolyzed per mg. of dry weight of tissue extract.

Kidney cortex		Brain		Liver		Intestinal mucosa*	
Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
0.240	0.571	0 026	0 028	0.017	0.014	0.318	0.510
0.650	0.206	0 027	0 035	0.016	0.012	0.650	0.288
0.520	0.389	0 038	0 031	0.014	0.010	0.360	0.751
0.656	0 487	0.029	0 030	0 018	0 010	0 384	0 353
0.469	0.278	0.028	0 019	0 014	0.016	0.421	0 563
0.488	0.190	0.036	0 024	0.012	0 015	0 632	0.609
0.722	0.305	0.033	0.021	0.014	0.010	0.829	0.176
0.515	0.252	0.027	0.017	0.014	0.010	1.335	0.459
0.433	0.289	0 024	0.024	0.012	0.012	0.265	0.568
0.592	0.252	0.027	0.023	0.011	0.012	0.551	0.329
Average 0.528	0 322	0 030	0.025	0.014	0.012	0 574	0.461
±0.031	±0 025	±0 0009	±0.0012	±0 0005	±0 0005	±0.072	±0 035

* 2 hours only.

likely therefore that ascorbic acid plays an important rôle in regulating phosphatase activity in the types of tissues examined.

Succinic Dehydrogenase—A study of the succinic dehydrogenase activity in normal and scorbutic guinea pig tissue was undertaken for several reasons: (a) It had been shown that sulfhydryl groups, indispensable for the activity of the enzyme, were maintained in the reduced form by biologically active reducing materials. (b) The succinate-fumarate system is generally recognized as an important hydrogen transport system in cellular respiration.

(c) We had previously observed from studies with tissue slices (25) that the respiratory behavior of scorbutic tissue deviated markedly from the normal.

Experiments were carried out by the Thunberg technique, in which methylene blue was used as the hydrogen acceptor. From the amount of hydrogen required for the reduction of a given amount of methylene blue, the enzyme activity was calculated as c.mm. of hydrogen transferred per mg. of tissue (dry weight) per hour. The value has been designated as Q_{H_2}, since it is analogous to the Q_{O_2} commonly used for expressing oxygen consumption.

Experiments were performed in duplicate in Thunberg tubes containing 1.0 ml. of enzyme preparation, 0.25 ml. of 0.001 M methylene blue, 1.25 ml. of M/15 phosphate buffer, pH 7.3, and, when necessary, enough water to make a final volume of 3.0 ml. After being evacuated, the tubes were sealed and placed in a water bath at 37.2°. After 5 minutes, 0.5 ml. of 0.5 M succinate (pH 7.3) was added from the hollow stopper. The time required for decoloration of 90 per cent of the methylene blue was noted by comparing the color of the experimental tubes with that of a tube containing 0.25 ml. of 0.0001 M methylene blue and heat-inactivated enzyme. A control tube with no substrate was used to determine the extent of reduction by the enzyme preparation alone. The results were discarded if the dye in the control tube was noticeably discolored in less than 3 hours.

Homogenized heart muscle containing a few drops of toluene was dialyzed against running water for 24 hours. The dialyzed tissue was collected and suspended in M/15 phosphate buffer (pH 7.3), so that 1.0 ml. of the preparation was equivalent to 50 mg. of fresh tissue.

Table III shows the succinic dehydrogenase activity of the heart from normal and scorbutic animals. The agreement of the Q_{H_2} values when 1 and 2 ml. of tissue suspension were used indicates that the rate of oxidation of succinate was proportional to the amount of tissue present. The dehydrogenase activity of the scorbutic heart was 20 per cent lower than that of normal tissue.

In measuring the dehydrogenase activity of skeletal muscle, the thigh muscle of guinea pigs was finely chopped and ground to a paste with sand and M/15 K_2HPO_4. After centrifugation of the

sand and tissue débris, the supernatant was mixed with an equal volume of 0.1 M acetate buffer (pH 4.5). The precipitate thus obtained was centrifuged, washed with a small volume of K_2HPO_4-acetate mixture, centrifuged again, and finally suspended in phosphate buffer, pH 7.3. 1 ml. of this preparation was equivalent to 500 mg. of the fresh muscle.

Table IV gives a summary of the results with preparations from the thigh muscle of normal animals, animals on a low vitamin level (assay), and severely scorbutic animals. Dehydrogenase

TABLE III

Succinic Dehydrogenase Activity of Heart Tissue

Q_{H_2} = c.mm. of H_2 per mg. per hour; T 37.2°; pH 7.3; anaerobic; methylene blue, M/12,000.

Normal		Scorbutic	
1 ml.	2 ml.	1 ml.	2 ml.
0.8	0.9	1.2	0.9
0.7	0.9	0.6	0.7
1.0	1.1	1.9	2.0
1.0	0.8	1.3	1.1
0.9	0.8	0.7	0.6
1.9	1.5	0.5	0.5
1.8	1.6	0.5	0.5
1.2	0.7	0.6	0.4
0.5	0.4	0.4	0.4
		0.3	0.3
Average .. 1.1 ±0.10	1.0 ±0.08	0.8 ±0.10	0.7 ±0.10
Total average. ... 1.0 ±0.07		0.8 ±0.07 (−20%)	

activity was decreased markedly during the development of scurvy. The average Q_{H_2} values for animals on a low vitamin level (minimum protective dose) and for severely scorbutic animals were 3.4 and 1.7 respectively, representing 29 and 65 per cent decreases below the normal Q_{H_2}, 4.8.

Cytochrome Oxidase—Cytochrome c was prepared from beef heart by the method of Keilin and Hartree (26). The method of Stotz, Sidwell, and Hogness (27) was used in determining the cytochrome oxidase activity of skeletal muscle (thigh), heart, and brain of normal and scorbutic guinea pigs. The tissues were homogenized in M/15 phosphate buffer (pH 7.2) and diluted

so that 1.0 ml. of the final preparation contained 10 mg. of either heart or skeletal muscle or 20 mg. of brain. The Q_{oxidase} (c.mm. of oxygen consumed per hour per mg. of dry tissue) was calculated from the constant rate of oxygen consumption over a 20 minute period. All values were corrected for the oxygen consumption of a control containing no added cytochrome. In the range of concentrations used, the oxygen consumption was proportional to the amount of tissue. It is obvious that the value reported

TABLE IV

Succinic Dehydrogenase Activity of Skeletal Muscle

Q_{H_2} = c.mm. of H_2 per mg. per hour; T 37.2°; pH 7.3; anaerobic; methylene blue, M/12,000.

Normal		Low vitamin level		Scorbutic	
1 ml.	2 ml.	1 ml.	2 ml.	1 ml.	2 ml.
4.3	4.0	3.3	2.8	2.2	2.1
3.3	3.2	3.1	2.6	3.0	3.0
4.9	3.7	3.7	3.1	1.4	1.3
4.0	3.4	2.3	2.1	1.6	1.2
3.9	3.7	3.8	3.2	2.4	1.8
5.2	3.2	1.8	1.6	2.6	2.6
7.8	5.1	4.8	3.6	1.7	1.4
5.5	4.0	3.5	3.3	0.9	0.6
6.6	5.5	5.0	4.2	1.7	1.1
7.8	7.1	4.8	4.9	0.8	0.7
				1.4	1.3
Average.. 5.3	4.3	3.6	3.1	1.8	1.6
±0.30	±0.25	±0.22	±0.19	±0.13	±0.16
Total average .4.8 ±0.22		3.4 ±0.15 (−29%)		1.7 ±0.10 (−65%)	

is lower than the true value because of the activity of cytochrome normally present in the tissue.

The results of our experiments on normal and scorbutic tissue are given in Table V. Although there was a wide variation in the Q_{oxidase} of tissues from different animals in the same group, there was a definite tendency toward decreased oxidase activity in scorbutic heart and skeletal muscle. The oxidase activity of scorbutic brain was not significantly different from that of normal brain. Phillips, Stare, and Elvehjem (28) have reported a decreased "indophenol oxidase" (cytochrome oxidase) activity of

the liver in both fluorosis and scurvy. In the case of cytochrome oxidase, there is no apparent reason to associate the decreased activity with a specific decrease in sulfhydryl groups, as in the case of succinic dehydrogenase and the tissue protease reported by Fomin and Romanyuk (29).

It is interesting to note that although scurvy is accompanied by an increased total body respiration and an increased respiration of tissue slices, there is a distinct decrease in the activity of two of the major respiratory enzymes (succinic dehydrogenase and cytochrome oxidase).

TABLE V

Cytochrome Oxidase Activity of Skeletal Muscle, Heart, and Brain

$Q_{oxidase}$ = c.mm. of O_2 absorbed per mg. of dry tissue per hour; T 37.2°; pH 7.3; air atmosphere in vessels.

Skeletal muscle		Heart tissue		Brain	
Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
68	48	138	120	41	42
53	38	110	52		27
82	51	222	94		24
147	53	101	52	21	20
46	30	99	100	14	25
42	40	88	42	39	43
110	35	100	34	20	39
74	38	117	24	22	20
35	46	134	63	42	31
53	14	90	92	35	37
Average... 71	39	120	67	29	31
±6.8	±2.3	±8.0	±6.5	±2.5	±2.0

SUMMARY

1. The effect of ascorbic acid deficiency on two hydrolytic and two respiratory enzymes in guinea pigs was studied.

2. Liver esterase activity decreased progressively with vitamin depletion to −65 per cent in acute scurvy. The properties of the enzyme did not afford evidence that ascorbic acid was present as a part of the enzyme, however, as claimed by one group of authors.

3. The phosphatase activity of intestinal mucosa and kidney cortex was changed only to a moderate degree during scurvy; the change in liver and brain was even less marked.

4. There was a marked drop in succinic dehydrogenase activity of heart and skeletal muscle parallel with ascorbic acid depletion.

5. Cytochrome oxidase showed a moderate decrease in activity in ascorbic acid-deficient heart and skeletal muscle tissue.

BIBLIOGRAPHY

1. Hellerman, L., *Physiol. Rev.*, **17**, 454 (1937).
2. King, C. G., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 137 (1940).
3. Kraut, H., and Pantschenko-Jurewicz, W., *Biochem. Z.*, **275**, 114 (1934).
4. Pantschenko-Jurewicz, W., and Kraut, H., *Biochem. Z.*, **285**, 407 (1936).
5. Kertesz, Z. I., *Ark. Kemi, Mineral. o. Geol.*, **12 B**, 4 (1938).
6. Strachitskiĭ, K. I., and Meerzon, T. I., *Biokhimiya*, **4**, 60 (1939).
7. Scoz, G., Cattaneo, C., and Gabrielli, M. C., *Enzymologia*, **3**, 29 (1937).
8. Raabe, S., *Biochem. Z.*, **299**, 141 (1938).
9. Giri, K. V., *Z. physiol. Chem.*, **245**, 185 (1937); **254**, 126 (1938).
10. Giri, K. V., *Biochem. J.*, **33**, 309 (1939).
11. von Euler, H., and Svenson, T., *Ark. Kemi, Mineral. o. Geol.*, **11 B**, 6 (1934).
12. Smith, J., and Maizels, M., *Arch. Dis. Childhood*, **7**, 149 (1932).
13. Smith, J., *Arch. Dis. Childhood*, **8**, 215 (1933).
14. Thannhauser, S. J., Reichel, M., and Grattan, J. F., *J. Biol. Chem.*, **121**, 697 (1937); *Biochem. J.*, **32**, 1163 (1938).
15. King, E. J., and Delory, G. E., *Biochem. J.*, **32**, 1157 (1938).
16. Hopkins, F. G., and Morgan, E. J., *Biochem. J.*, **32**, 611 (1938)
17. Hopkins, F. G., Morgan, E. J., and Lutwak-Mann, C., *Biochem. J.*, **32**, 1829 (1938).
18. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).
19. Rapkine, L., and Trpinac, P., *Compt. rend. Soc. biol.*, **130**, 1516 (1939).
20. Ecker, E. E., Pillemer, L., Martiensen, E. W., and Wertheimer, D., *J. Biol. Chem.*, **123**, 351 (1938).
21. Knaffl-Lenz, E., *Arch. exp. Path. u. Pharmakol.*, **97**, 242 (1923).
22. Glick, D., and King, C. G., *J. Biol. Chem.*, **94**, 497 (1931-32).
23. Kay, H. D., *Biochem. J.*, **22**, 855 (1928).
24. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
25. Stotz, E., Harrer, C. J., Schultze, M. O., and King, C. G , *J Biol. Chem.*, **120**, 129 (1937).
26. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
27. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, **124**, 733 (1938).
28. Phillips, P. H., Stare, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, **106**, 41 (1934).
29. Fomin, S. V., and Romanyuk, N. M., *Ukrain. Biokhem. Zhur.*, **9**, 355 (1936); *Chem. Abst.*, **31**, 2660 (1940).

A COMPARISON OF KETOSIS IN MAN AND DOG

By LATHAN A. CRANDALL, Jr.

(From the Department of Physiology, University of Tennessee College of Medicine, Memphis)

It is common knowledge that the intensity of ketosis is greater in man than in the dog. The question arises whether the difference is one of degree or of kind. The rate of development of ketosis, blood and urine acetone body concentration, and response to oral glucose have therefore been compared in the two species in order to determine whether such information would or would not suggest the presence of a qualitative difference in ketone body metabolism.

Methods

The men used in these experiments were freshman medical students. They were fasted for 3 days, during which blood samples were taken; the urine of the final day was collected and analyzed. In six instances the fast was terminated by the oral administration of 70 gm. of glucose, and blood acetone bodies were determined at intervals for 2 hours.

The dogs were angiostomized animals studied in connection with problems that have been reported (1, 2). Ketosis was produced in the dogs by periods of fasting or fasting followed by fat feeding, and was terminated in six animals by 20 gm. of glucose orally.

Total acetone bodies were determined in the blood samples by the method of Crandall (3), and in urine by the same method after removal of interfering substances by precipitation with copper sulfate and calcium hydroxide, the final dilution of the urine being 1:50 or 1:100; the results are expressed as β-hydroxybutyric acid. Acetone and diacetic acid were determined by the same method except that potassium dichromate was not added and the

period of refluxing was reduced to 40 minutes. β-Hydroxybutyric acid was determined by difference, the validity of this calculation having been checked on a number of samples by removal of the acetone and acetoacetic acid by acid hydrolysis and open boiling, and determination of the remaining β-hydroxybutyric acid by the usual method. Calculated and directly determined β-hydroxybutyric acid agreed within the limits of error of the method.

Results

The onset of ketosis in man is abrupt and its development rapid, as can be seen from Table I. This may be compared with

TABLE I

Rate at Which Ketosis Develops during Fasting in Man

The values for acetone bodies are expressed as mg. of β-hydroxybutyric acid per 100 ml. of blood

Subject	Fasting		
	15 hrs.	39 hrs.	63 hrs.
Ne . ..	0.6	32.8	43.6
Ro..	0.8	6.3	15.8
Ja..	0.6	21.5	24.9
Do ...	0 4	29.6	47.1
Sh.	0.6	24.8	60.5
Al......	0 5	2.5	36 8

observations on dogs presented by Crandall, Ivy, and Ehni (2), in which ketogenesis was determined by analyses of inflowing and outflowing hepatic blood. Blood acetone body concentrations in the postabsorptive state are within the same range in the two species, but ketosis in the dog does not appear until 48 to 72 hours have elapsed after the last meal and then increases slowly. Blood acetone body concentrations in the venous arm blood of man are higher after 39 hours of fasting than any arterial blood concentrations produced by fasting up to 11 days in the dog. Only after 5 to 11 days of fasting followed by 3 to 9 days of exclusive fat feeding is canine ketosis comparable to that of the man fasted 39 to 63 hours. The individual variations in human ketosis should be noted; development was slower in Subjects Ro and Al

than in the other subjects. This could not be correlated with body build or amount of adipose tissue; Subject Sh, who attained the highest concentration of acetone bodies in his blood, was especially tall and slender.

As shown in Table II, the per cent of the total ketone bodies in the form of β-hydroxybutyric acid is, on the average, slightly higher in the dog than in man. Systemic venous samples were not taken from the dog; the comparison is therefore made between canine arterial and human venous samples. It is believed that this does not influence the relative proportions of the acetone

TABLE II

β-Hydroxybutyric Acid As Per Cent of Total Blood Acetone Bodies in Dog and Man

Dog No.	Total arterial acetone body concentration	β-Hydroxy-butyric acid	Subject	Total venous acetone body concentration	β-Hydroxy-butyric acid
	mg per 100 ml.	*per cent*		*mg per 100 ml.*	*per cent*
1	1.6	63	M	37.4	60
2	4.7	91	F	55.5	62
3	8 1	89	F₁	13.0	68
2	11.6	72	Po	44 5	60
3	9.6	74	Sh	51.0	80
4	31.4	60	Sh	64.1	62
5	40.0	71	Do	47.1	59
4	27.2	64	Sh	60.5	61
5	12.2	73	Al	36.8	66
Average.		73			64

bodies, since Crandall (1) has shown that these substances are produced by the liver and must be utilized by the tissues in substantially the same proportions that are found in arterial blood. The slight difference between the two species in the per cent of the total that is β-hydroxybutyric acid does not suggest any essential difference in acetone body metabolism.

The ratio of the concentration of acetone bodies in blood to their concentration in urine is compared for man and dog in Table III. In the dog the urine contains from 7 to 62 times as many acetone bodies per unit volume as does the blood; similar figures for man are 8 to 23. However, the urine concentration is more than 19 times the blood concentration in only two out of

nine experiments in the dog. The ketone bodies are evidently low threshold substances in both species. The urine volume will be a major factor determining the amount excreted in the urine per day at any given blood level. From these experiments also we can derive no indications of a qualitative difference between the two species. The greater ketonuria of man is dependent on the higher blood concentration.

A final comparison of the two species has been made by following the rate at which the blood acetone body concentration decreases after the oral administration of glucose (Table IV). The response is more prompt in the dog than in man, a fall being appar-

TABLE III

Total Acetone Body Urine to Blood Concentration Ratios of Dog and Man

The acetone body concentrations are expressed as β-hydroxybutyric acid in mg. per 100 ml.

Dog No.	Arterial blood concentration	Urine concentration	Ratio, urine to blood	Subject	Venous blood concentration	Urine concentration	Ratio, urine to blood
4	27.2	240	9.2	Mi	37.4	615	16.5
5	12.2	183	15.0	Fr	55.5	425	7.7
1	10.2	75	7.4	Sh	51.0	740	14.5
1	3.4	69	20.4	Sh	64.1	820	12.8
1	3.8	211	55.5	Ne	43.6	805	18.5
3	5.8	55	9.5	Ro	15.8	180	12 5
4	3.1	193	62 4	Ja	24.9	222	8.9
4	1.7	32	18.8	Do	47.1	537	11.4
3	6.1	108	17 8	Sh	60.5	1380	22.8
				Al	36.8	492	13.4

ent within 30 minutes in every canine experiment and in but two out of five human experiments. Once the acetone bodies begin to diminish, however, the rate of decrease in the human experiments is similar to that in the dog. The two human subjects (Subjects Fr and Po) in whom there was any fall in the first half hour showed decreases of 7 and 9 mg. per 100 ml. per 30 minutes as compared with 8, 7, and 8 for the three dogs in which ketosis had not been completely abolished within this period. Subject Sh showed a drop of 10 mg. per 100 ml. in 45 minutes, and Dog 1 a drop of 9 mg. for the same period. Subjects Sh, Mi, Fr, Fi, and Po show decreases of 8, 8, 11, 10, and 20 mg. per 100 ml. of blood for the second 30 minutes; this is somewhat more rapid than

the rate of decrease in the dogs for the first 30 minute period. There is nothing about the manner in which the blood acetone body concentrations change after the administration of glucose to suggest a difference in the mechanisms of ketosis in the two species.

Table IV also shows data on β-hydroxybutyric acid as per cent of total acetone bodies before and after glucose. In both dog and

TABLE IV

Effect of Oral Administration of Glucose on Fasting Ketosis in Man and Dog

"Total" refers to mg. of total acetone bodies (expressed as β-hydroxybutyric acid) per 100 ml. of blood; "per cent of total" refers to the per cent of total blood acetone bodies present as β-hydroxybutyric acid.

Subject	Fasted	Days fat fed	Acetone bodies in human blood												
			0 min.		15 min.	30 min.		45 min.		60 min.		90 min.		125 min.	
			Total	Per cent of total	Total	Total	Per cent of total	Total	Per cent of total	Total	Per cent of total	Total	Per cent of total	Total	Per cent of total
	hrs.														
Sh	69		51.0	74		50.4	67			42.9	56	30.1	51		
Sh	40		64.1	62				56.4	46			38.8	43	22.8	30
Mi	72		37.4	60		38.2	54			30.0	45	18.5	47		
Fr	72		55.5	62		48.9	52			37.0	42	24.4	32		
Fi	72		13.0	68		14.2	68			4.6	71	2.5	64		
Po	72		44.5	60		35.8	62			16.0	49	6.9	46		
Dog No.			Acetone bodies in dog blood												
	days														
3	6	11	27.2	64		19.4	60			13.3	52				
2	11	6	9.6	74		2.9	55			2.5	35				
1	11	7	11.6	72				2.8	51			1.0	10		
4	7	3	10.2		6.1	2.5									
2	6	0	5	8		0.7						0.5			
1	6	0	3.1			0.7						0.6			

man one can observe a trend toward a decreasing proportion of β-hydroxybutyric acid as the total concentration falls.

DISCUSSION

As an explanation for ketosis, Crandall, Ivy, and Ehni (2) have offered the hypothesis that the acetone bodies can replace glucose

in at least some phases of metabolism, that acetone body production therefore decreases the demand for hepatic glucose production, and that ketosis may be regarded as a special type of metabolism (since ketogenesis is not an essential part of fat oxidation) occurring in the glucoprivic state. The experiments leading to this hypothesis were performed on dogs. Since the hepatic ketone body output cannot be determined in man, it has seemed worth while to make such comparisons between human and canine ketosis as are possible. The two species appear to differ only in the rate of onset and intensity of ketonemia. They are comparable with regard to the proportions of the various acetone bodies present in the blood, the proportion excreted in the urine, and the rate at which ketonemia decreases after the oral administration of glucose. The data indicate no differences in type of acetone body metabolism in the two species. In the dog the concentration of acetone bodies in the blood has been correlated with the rate of production by the liver (2). If this holds true for the human also, ketone body oxidation may account for a greater fraction of the total metabolism of fasting in this species.

SUMMARY

The ketosis of fasting in dog and man has been compared on the basis of the concentration of acetone bodies in blood and urine, rate of development, proportion of different acetone bodies in blood, and response to glucose. Ketonemia develops within 39 hours in man, as compared to 2 to 3 days in the dog, and attains considerably higher values in the former species. In other respects human and canine ketosis are comparable, suggesting that there is no fundamental difference in acetone body metabolism between these two species.

BIBLIOGRAPHY

1. Crandall, L. A., Jr., *J. Biol. Chem.*, **135**, 139 (1940).
2. Crandall, L. A., Jr., Ivy, H. B., and Ehni, G. J , *Am. J. Physiol.*, **131**, 10 (1940).
3. Crandall, L. A., Jr., *J. Biol. Chem.*, **133**, 539 (1940).

THE EFFECT OF CONCENTRATION ON THE RATE OF UTILIZATION OF β-HYDROXYBUTYRIC ACID BY THE RABBIT

By ARNE N. WICK and D. R. DRURY

(From the Scripps Metabolic Clinic, La Jolla, California, and the Department of Physiology, School of Medicine, University of Southern California, Los Angeles)

Our attitude towards the significance of the ketone bodies in metabolism has undergone considerable change in recent years. At one time the presence of any appreciable amount of these substances in the blood stream would have been regarded as an indication of impaired metabolism. Today we look on such an occurrence as perfectly natural and resulting from a normal mechanism for utilizing fatty acids which consists of a partial oxidation of these substances to form ketone bodies by the liver and distribution of these ketone bodies to the tissues by the blood for complete combustion (1, 2).

Our concern in the work to be presented in this paper is with the effect of blood ketone concentration on the rate of utilization by the tissues. Results have appeared which suggest that the rate of utilization is some function of the concentration of these substances (2, 3). The work of Dye and Chidsey (4) points definitely to some such relationship, although they report utilization rate against amount injected and not actual concentration. Also they did not maintain any particular level of concentration during any one experimental period but rather had a very extended range of concentrations in each test run. Their results indicate that the greater the amount of ketone bodies injected the greater the utilization. In our work we aimed to bring the blood ketone concentration to a desired level and then maintain it there during the period that utilization was measured.

Methods

Rabbits of body weight between 2 and 3 kilos were used. A solution of the sodium salt of β-hydroxybutyric acid[1] dissolved in saline was injected into the marginal ear vein after denervation of this skin area.   The rate of injection was rapid for the first 15 to 20 minutes in order to attain the desired level.   Without making any other changes the rate was then slowed down to that which we had decided to use for a subsequent level and was maintained for 10 minutes in order to attain equilibrium conditions before starting the period of observation.   The urinary bladder was then emptied and a blood sample taken for total ketone body determination which was made by the Barnes and Wick method (5).   β-Hydroxybutyric acid, acetoacetic acid, and acetone were determined together and were calculated and are expressed as β-hydroxybutyric acid.   The rate was then maintained for a period of 20 to 30 minutes and then another blood sample was taken and the urine formed during this period was collected for ketone body determination.   (Unless there was little change in the blood ketone concentration between the beginning and end of the period, the experiment could not be used, since it would mean that the concentration had not been constant and also that a somewhat large correction would have to be made for the change in the amount of ketone in the tissues of the animal during the run.)   A fair number of determinations were therefore eliminated, especially at the beginning of our study, but we soon learned by trial and error and at the end were able to regulate conditions so that few runs had to be discarded for this reason.   The estimation of the acetone body utilization during the period was made by subtracting the urinary excretion from the amount injected and then making a correction for the small change in concentration of the substance in the tissues of the body.   This latter was made on a basis of assuming that 50 per cent of the body weight was water that diluted the ketone bodies.   The procedure may be illustrated by the protocol of a typical experiment.

Rabbit—Weight 2450 gm.; injected with a solution of sodium β-hydroxybutyrate containing 3.25 gm. per cent of β-hydroxybutyric acid.   1.50

---

[1] A very pure preparation of the racemic salt obtained from The British Drug Houses, Ltd., was employed in all of the experiments reported in this paper.

p.m. injection started. 2.13 p.m. 92 cc. of solution have been injected, and the rate of injection was slowed to 1.24 cc. per minute. 2.26 p.m. 16.5 cc. have been injected since 2.13, the bladder was emptied, the urine discarded, and a blood sample taken (containing 207 mg. per cent of β-hydroxybutyric acid). 2.46 p.m. 24.8 cc. have been injected since 2.26, a blood sample was taken (203 mg. per cent of β-hydroxybutyric acid), and urine collected (194 mg. of β-hydroxybutyric acid).

The mean value of the concentration of β-hydroxybutyric acid in the blood during the experimental period = 205 mg. per cent.
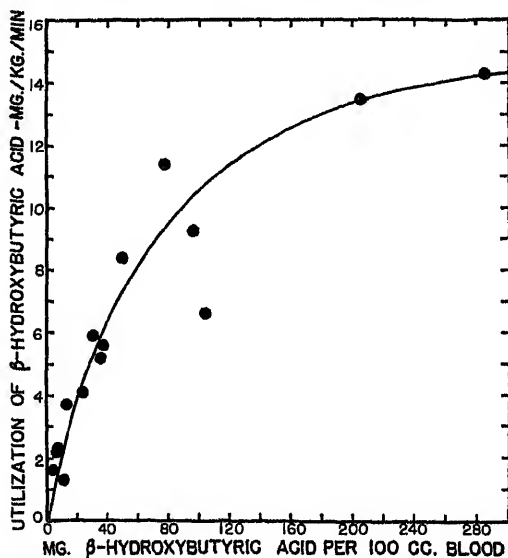
Fig. 1. The effect of increases in the concentration of β-hydroxybutyric acid in the blood on the utilization rate of this substance by the animal.

Calculation of Utilization Rate

β-Hydroxybutyric acid injected = 24.8 × 32.5 mg. = 805 mg.
Correction for change in concentration in tissues =
$$\frac{(207-203) \text{ mg.}}{100} \times \frac{2450}{2} = +49 \text{ mg.}$$
Amount excreted by kidneys = −194 mg.

Utilization rate = $\dfrac{(805 - 194 + 49) \text{ mg.}}{20 \text{ minutes} \times 2.45 \text{ kilos}}$ = 13.5 mg. per kilo per minute

Fifteen satisfactory determinations were carried out in this manner but at various blood ketone levels. The results are given in Fig. 1. It is apparent that there is a very definite increase

in utilization rate with increased concentration in the blood.   The curve depicting this relationship rises very sharply at first and tends to flatten out at a concentration around 80 mg. per cent, and the rate of rise becomes less and less above this point.   At the very high levels (above 200 mg. per cent) the rate of utilization is so high that almost all the energy requirements of the animal must be supplied by the burning of the ketones.   In two of the animals studied at the very high levels we determined the oxygen consumption and found it to be 13.45 and 15.15 cc. per kilo per minute.   The amounts of hydroxybutyric acid disappearing at these times would require 85 to 90 per cent of the oxygen used. Similar percentages in the heart-lung preparation were obtained by Barnes et al. (3) with high concentrations of β-hydroxybutyric acid.   It would appear then that the metabolic rate of the animal would be the limiting factor for the ketone utilization rate at these high levels; that is to say, that almost all the energy needs of the body were then supplied by ketone body oxidation, so that this latter could not be further increased without an increase in metabolic rate (6).

DISCUSSION

Recent work indicates that there is no difference in the capacity of diabetic and of normal tissues to utilize ketone bodies (2, 4, 7). It is reasonable to assume then that the relationship between concentration and utilization which we have found in normal animals will be true for diabetes.   The ketonemia which occurs in diabetes and other "ketosis" states would therefore result from a high rate of production of these substances by the liver and would give rise to a correspondingly high rate of utilization of them by peripheral tissues.   This agrees with the utilization rates determined by the arteriovenous difference method (2); in diabetic animals with blood ketone body concentrations over 40 mg. per cent the fraction of metabolism taken care of by acetone body oxidation was about 75 per cent.

Our results have a bearing on the question of whether all fat burned by the peripheral tissues must be first changed by the liver to ketone bodies.   The view has been presented (8) that this is a necessary step in the combustion of fatty acids.   It can be readily seen, however, that a very high utilization of ketone bodies by

the peripheral tissues is only attained when the concentration of them in the blood becomes very high; that is, at levels rarely if ever seen in actual life. A normal individual will derive about 90 per cent of his energy from the combustion of fat after a 48 hour fast or on a high fat diet. Under such conditions he would ordinarily show a moderate ketonemia of 20 to 40 mg. per cent. At such concentrations only about half of the total metabolism of the tissues would be taken care of by ketone body oxidation; the remainder of the fat oxidized would presumably be burned directly in the tissues.

On the other hand the rate of ketone body utilization at the blood levels of ordinary ketosis must be so high that we cannot explain ketone body production by the liver as due to incomplete catabolism of the end-products of β oxidation in this organ (9) or as a by-product of glyconeogenesis from fat (10). The fatty acid molecule worked over by the liver under these circumstances must be very largely if not completely converted into ketone body molecules in order to account for the large production of these that would be necessary for the consumption of the tissues (11). This supports the view already presented in reports from these laboratories (2) that the conversion of fatty acid to ketone bodies by the liver is an important mechanism for the preparation of it for oxidation by the other tissues; it is a normal metabolic process and not secondary to other reactions. It may be considered analogous to the conversion in the liver of amino acids to glucose for utilization by the tissues.

SUMMARY

The utilization rate for β-hydroxybutyric acid in the intact animal is dependent on the concentration of it in the blood. The upper limit of utilization seems to be reached when the oxidation of this substance uses about 90 per cent of the oxygen consumed by the animal.

BIBLIOGRAPHY

1. Lundsgaard, E., *Harvey Lectures*, **33**, 85 (1937–38).
2. Barnes, R. H., and Drury, D. R., *Proc. Soc. Exp. Biol. and Med.*, **36**, 350 (1937). Barnes, R. H., Drury, D. R., Greeley, P. O., and Wick, A. N., *Am. J. Physiol.*, **130**, 144 (1940).
3. Barnes, R. H., MacKay, E. M., Moe, G. K., and Visscher, M. B., *Am. J. Physiol.*, **123**, 272 (1938).

4. Dye, J. A., and Chidsey, J. L., *Am. J. Physiol.*, **127,** 745 (1939).
5. Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, **131,** 413 (1939).
6. Mirsky, I. A., and Broh-Kahn, R. H., *Am. J. Physiol.*, **120,** 446 (1937).
7. Chaikoff, I. L., and Soskin, S., *Am. J. Physiol.*, **87,** 58 (1928).
8. Blixenkrone-Møller, N., *Z. physiol. Chem.*, **253,** 261 (1938).
9. Mirsky, I. A., *Am. J. Physiol.*, **115,** 424 (1936).
10. Soskin, S., Mirsky, I. A., Zimmerman, L. M., and Crohn, N., *Am. J. Physiol.*, **114,** 110 (1935-36).
11. MacKay, E. M., Barnes, R. H., Carne, H. O., and Wick, A. N., *J. Biol. Chem* , **135,** 157 (1940).

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXIII. THE FATTY ACIDS IN THE PHOSPHATIDE PREPARED FROM CELL RESIDUES FROM TUBERCULIN*

By ROBERT L. PECK† AND R. J. ANDERSON

(*From the Department of Chemistry, Yale University, New Haven*)

It was shown in the first analysis of the phosphatide isolated from the human tubercle bacillus, Strain H-37 (1), that the fatty acids were a mixture of solid saturated, liquid unsaturated, and liquid saturated acids. The solid saturated acid was found to be mainly palmitic acid and the liquid unsaturated acid was mainly oleic acid. These ordinary acids, therefore, do not present anything of special interest, either chemically or biologically.

The liquid saturated fatty acids are, however, of particular importance because they are specific metabolic products of the tubercle bacillus and they possess certain important biological properties (2). Owing to inadequate material it was impossible to make a complete investigation of the liquid saturated acids but it was found that they had a high molecular weight and that they showed a dextrorotation. By means of the pseudothiourea salt it was possible to separate the acids into an optically inactive fraction and a fraction that possessed a higher dextrorotation than the original material. On the basis of this evidence it was assumed that the liquid saturated acids of the phosphatide were a mixture of tuberculostearic acid and phthioic acid similar to that which occurs in the acetone-soluble fat (3).

In view of the unusual physical and physiological properties of the liquid saturated acids it was desirable to investigate them

more thoroughly, but for this purpose larger quantities of phosphatide were required than were available.

Recently in an investigation of the lipids contained in cell residues, which are a by-product in the preparation of tuberculin, we obtained considerable quantities of phosphatide which appeared to be identical with the phosphatide analyzed previously (1) but investigation of the polysaccharide component (4) showed that it was not identical with the polysaccharide contained in the phosphatide prepared from living bacilli.

The present report deals with the composition of the fatty acids obtained from the phosphatide used in the polysaccharide investigation. The results obtained indicate that the component fatty acids were similar to those found in the first analysis (1). The solid saturated acids consisted almost entirely of palmitic acid, while the presence of oleic acid was indicated by the low iodine number of the mixed liquid acids and by the formation of stearic acid on catalytic reduction. The liquid saturated fatty acids consisted principally of tuberculostearic acid (3, 5) but a small amount of higher branched chain acids possessing a low dextrorotation was isolated. The latter acids could not be obtained in pure form and probably represented a mixture of homologues of phthioic acid. No pure phthioic acid (6) was found.

<div align="center">EXPERIMENTAL</div>

The phosphatides had been saponified and the fatty acids had been isolated as described in Paper LXI (4) of this series. In addition to the two lots of phosphatide referred to in the report mentioned above, a third phosphatide fraction, also prepared from cell residues, had been saponified and the fatty acids had been isolated exactly as described in the paper mentioned above.

The fatty acids from the three lots of phosphatide were separated by the lead salt-ether procedure into solid and liquid acids. The iodine numbers of the crude liquid acids were low, thus indicating a large proportion of liquid saturated fatty acids. Data concerning the fatty acids are given in Table I.

The Solid Saturated Fatty Acids—The fatty acids isolated from the ether-insoluble lead salts were a solid crystalline mass at room temperature. On treatment with warm methyl alcohol a small

amount of insoluble oily droplets appeared which was removed by filtration. The alcohol-insoluble substance which amounted to 0.7 per cent of the solid acids was apparently mycolic acid. It separated from hot acetone, on cooling, in the form of fine globular particles, m.p. 55–56°.

The fatty acids contained in the methyl alcoholic solution were esterified by adding dry hydrochloric acid and refluxing for 5 hours. The esters were isolated and fractionated through a modified Widmer column. The first fractions, amounting to 82 per cent of the total esters, melted between 26–28° and represented practically pure methyl palmitate. The ester was saponified and the free acid was isolated and crystallized from acetone. The acid crystallized in colorless, irregular thin plates and possessed

TABLE I

Fatty Acids from Phosphatides Prepared from Cell Residues from Tuberculin

Phosphatide saponified	Total fatty acids	Solid fatty acids	Liquid fatty acids	Iodine No. of liquid fatty acids
gm.	*gm.*	*gm.*	*gm.*	
40.0	26.60	13.80	12.30	40.6
40.0	26.90	9.97	15.88	32.7
24.6	15.45	3.77	11.08	19.0

the properties of palmitic acid, m.p. 62–63°, molecular weight by titration 255.

The balance of the ester was fractionated through a Craig column (7) and gave mainly methyl palmitate. However, a small amount of a higher boiling fraction was obtained which apparently was slightly impure methyl stearate. After saponification the free acid was crystallized from acetone and it melted at 64–67°, molecular weight by titration 280.

From the results obtained it is evident that the solid saturated acids consisted principally of palmitic acid together with very small amounts of stearic acid and mycolic acid.

The Liquid Acids—The three lots of liquid acids were combined and subjected to catalytic reduction with hydrogen and platinum oxide but apparently some poison was present which prevented complete reduction. The acids were, therefore, converted into methyl esters and the latter were distilled *in vacuo*. The purified

esters were catalytically reduced and yielded a product which was completely saturated, since it absorbed no iodine.

The reduced esters were saponified and the free acids were separated by means of the lead salt-ether procedure into solid reduced acids and liquid saturated acids. The lead salt-ether treatment was repeated on the liquid saturated acids until the solid reduced acids were removed, as shown by the fact that the lead salts finally obtained were completely soluble in ether.

The Solid Reduced Acids—The acids isolated from the ether-insoluble lead salts, as described above, were converted into methyl esters and fractionated by distillation *in vacuo*. A small amount of forerun yielded on saponification an acid that melted at 51–52°, molecular weight by titration 263, thus indicating the presence of an acid lower than stearic acid. The main ester fraction was saponified and the free acid was crystallized from acetone. The acid melted at 69–70°, molecular weight by titration 284.4, and was, therefore, stearic acid.

In view of the low iodine number of the mixed liquid acids, we conclude that the chief constituent of the unsaturated acids was oleic acid.

The Liquid Saturated Fatty Acids. Isolation of Tuberculostearic Acid—The liquid saturated fatty acids obtained from the ether-soluble lead salts, after the solid reduced acids had been removed as mentioned above, were converted into methyl esters and the esters were fractionated in a high vacuum through a modified Widmer column. The first and principal fraction, 11.7 gm., corresponded in properties to methyl tuberculostearate. The ester was a colorless oil that distilled rapidly with the temperature of the oil bath at 180–187° and at 0.2 mm. pressure. It was saponified and yielded an acid which was a colorless, optically inactive oil, m.p. 10–12°, molecular weight by titration 299.6.

Analysis—$C_{19}H_{38}O_2$ (298).   Calculated, C 76 51, H 12 75
Found,   " 76.17, " 12.64

The tribromoanilide was prepared according to Robertson (8). The product was recrystallized from methyl alcohol after the solution had been decolorized with norit. Colorless needle-shaped crystals were obtained which melted at 93–94°. The melting point was the same as that reported by Spielman (5) for the tri-

bromoanilide of tuberculostearic acid. The yield was 51 per cent of the theoretical.

Isolation of a Higher Optically Active Liquid Saturated Fatty Acid —The residue which remained after the methyl tuberculostearate had been distilled off was transferred to a small fractionating flask. The ester distilled slowly at a temperature of the oil bath of 230–245° and at 0.001 mm. pressure. The ester was a colorless oil that weighed 0.7 gm.

Rotation—$[\alpha]_D$ in ether = +2.4°

The ester was saponified and the free acid, 0.65 gm., was obtained as a nearly colorless thick oil. It solidified at about 15° and melted at 18°.

Rotation—$[\alpha]_D$ in alcohol = +2.7°. Found, mol. wt. by titration 345
Analysis—Found, C 78.38, 78.15, H 12.87, 12.77

The values found would indicate that the acid was a mixture of higher branched chain acids analogous to phthioic acid.

It is evident from the results obtained that the liquid saturated fatty acids contained in the phosphatide from the bacterial residues consisted mainly of tuberculostearic acid. However, small amounts of higher optically active branched chain acids were also present. The nature of the latter acids could not be determined fully but no pure phthioic acid was found.

SUMMARY

1. An examination has been made of the fatty acids obtained from the phosphatide prepared from bacterial residues from the manufacture of tuberculin.

2. The solid saturated acids consisted mainly of palmitic acid but traces of stearic acid and mycolic acid were present.

3. The liquid unsaturated acid after catalytic reduction was essentially stearic acid and hence the principal unsaturated acid was oleic acid.

4. The liquid saturated fatty acids consisted mainly of tuberculostearic acid, $C_{19}H_{38}O_2$.

5. A small amount of higher branched chain dextrorotatory acids was present which were not definitely identified.

6. No pure phthioic acid was found.

BIBLIOGRAPHY

1. Anderson, R. J., *J. Biol. Chem.*, **74,** 537 (1927).
2. Sabin, F. R., Doan, C. A., and Forkner, C. E., *J. Exp. Med.*, **52,** suppl. 3, 22 (1930).
3. Anderson, R. J., and Chargaff, E., *J. Biol. Chem.*, **85,** 77 (1929-30).
4. Anderson, R. J., Peck, R. L., and Creighton, M. M., *J. Biol. Chem.*, **136,** 211 (1940).
5. Spielman, M. A., *J. Biol. Chem.*, **106,** 87 (1934).
6. Spielman, M. A., and Anderson, R. J., *J. Biol. Chem.*, **112,** 759 (1935-36).
7. Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **9,** 441 (1937).
8. Robertson, P. W., *J. Chem. Soc.*, **115,** 1210 (1919).

ISOLATION OF A NEW SULFUR-CONTAINING AMINO ACID (LANTHIONINE) FROM SODIUM CARBONATE-TREATED WOOL

By MILLARD J. HORN, D. BREESE JONES, and S. J. RINGEL

(From the Protein and Nutrition Research Division, Bureau of Agricultural Chemistry and Engineering, United States Department of Agriculture, Washington)

In a recent publication (1) the authors reported the isolation of a crystalline organic selenium compound from plant material grown on seleniferous soil. This compound agreed in its properties and composition with an isomorphous mixture of a sulfur and selenium amino acid having the formula,

$$HOOC—CH(NH_2)—CH_2—Se—CH_2—CH_2—CH(NH_2)—COOH$$
$$(S)$$
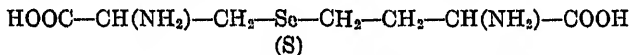
As far as we are aware, the only instance recorded of the isolation of a compound of this type is that of the thio ether amino acid which Küster and Irion (2) claimed to have obtained from the products of reaction of sodium sulfide solution on wool at room temperature, and to which they ascribed the formula,

$$HOOC—CH(NH_2)—CH_2—CH_2—S—CH_2—CH(NH_2)—COOH$$
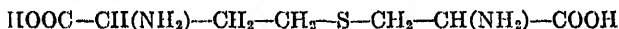
Subsequent attempts, however, on their part to isolate the compound were unsuccessful.

As the quantity of our selenium-sulfur compound was small, and the supply of the source material was exhausted and could not be replenished for several weeks, it was decided that in the meantime it would be worth while to repeat the work of Küster and Irion with the hope of isolating the thio ether from wool. Familiarity gained with its properties should be of value later when our work on the analogous selenium-sulfur compound is resumed.

Several attempts to isolate the thio ether by carefully following

Küster and Irion's procedure proved unsuccessful. Some departures from their technique were then followed in which was included the use of pyridine to precipitate the free amino acids from the acid hydrolysates. Five attempts were made with this procedure with 100 gm. of wool each time. In every case active cystine in the form of hexagonal plates was isolated, and also some crystals which were identified as inactive cystine. In one instance only was a small quantity of a crystalline substance isolated which showed the properties of a thio ether. Tests for disulfide and for sulfhydryl groups in this substance were negative. The crystals were similar in appearance to those of the symmetrical thio ether amino acid which is herein described. The quantity obtained, however, was too small to permit closer examination.

The fact that Küster and Irion were unsuccessful in repeating the isolation of their thio ether suggests that its formation may be limited to a very narrow range of conditions, which, if not rigidly controlled, would yield negative results. Slight changes in pH, temperature, duration of the reaction, and other factors may have a pronounced effect upon the nature of the end-products.

Hoffman (3) found that when hair was heated with 1 per cent Na_2CO_3 solution for a short time, it lost about 24 per cent of its sulfur content. The hair thus treated was identical in appearance to that which had not been treated, and although it retained about three-fourths of its original sulfur content, no cystine could be isolated from it. Consideration of Hoffman's results raised the question as to the type of linkage of the sulfur in the alkali-treated wool and suggested that the disulfide bonds may have been split in some manner such as occurs when wool is treated with sodium sulfide with the possible formation of thio ether amino acids.

Evidence has now been obtained showing that such is the case. When wool was boiled for a short time with dilute Na_2CO_3 solution, and then hydrolyzed with HCl, there was isolated from the hydrolysate a crystalline thio ether amino acid. This is not, however, the thio ether that Küster and Irion claimed to have isolated, but the symmetrical compound, β-amino-β-carboxy-ethyl sulfide (I). Two other compounds having the properties

of thio ether amino acids were also isolated, which were found to differ from (I) with respect to crystalline form and solubility. The fact that both had about the same nitrogen content as (I) suggests that the three may be optical isomers. They will be the subject of further study and the results will be published later. The isolation of these compounds from wool according to the same procedure has been repeated seventeen times.

EXPERIMENTAL

100 gm. of wool, freed mechanically from some extraneous material, were washed with cold water and then boiled for 1 hour with 1.5 liters of 2 per cent Na_2CO_3 solution. The Na_2CO_3-treated wool was then separated on cheese-cloth and washed with water. It is of interest to note that when the alkaline filtrate from the treated wool was acidified a strong odor of H_2S was observed. The treated wool was hydrolyzed by boiling with 200 cc. of concentrated HCl for 15 hours. After decolorization with norit the hydrolysate was then concentrated to a thick sirup *in vacuo*. In order to remove most of the free HCl the residue was taken up in about 100 cc. of 95 per cent alcohol and the solution was again concentrated as before. The thick, sirupy residue was dissolved in 600 cc. of absolute alcohol. A small quantity of NaCl which separated was removed by filtration, and the volume of the filtrate was made up to 1 liter with absolute alcohol. Pyridine was then cautiously added in small quantities with shaking until an additional drop caused no further precipitation. After the mixture had stood for about an hour the precipitate was separated by centrifugation and washed with absolute alcohol. It was then heated to boiling with 100 cc. of water until the greater part had dissolved. After cooling, the mixture was allowed to stand in a refrigerator overnight. The material which had separated was removed by filtration and redissolved by heating with 100 cc. of water to which 5 cc. of concentrated ammonia had been added. After standing for about half an hour the small quantity of a reddish, flocculent substance which had settled was removed by filtration. The clear filtrate was then concentrated by distillation *in vacuo*. As the ammonia was being removed, the thioamino acid separated. It was removed by filtration and recrystallized from dilute am-

monia by allowing the solution to stand exposed to the air until most of the ammonia was expelled. It separated in the form of thin plates (Fig. 1). About 0.5 gm. of this product was isolated. The other two compounds previously referred to, which are much more soluble, were obtained from the filtrate of (I). The joint yields obtained of these two compounds were about 0.5 gm.

Compound (I) had the following percentage composition.

$C_6H_{12}SN_2O_4$.   Calculated   C 34.59, H 5.81, N 13 46, S 15 41
              Found.   " 34 67, " 5.99, " 13.48, " 15 17

Tests for sulfhydryl and disulfide groups were negative. A strong positive test was obtained with ninhydrin reagent, indi-
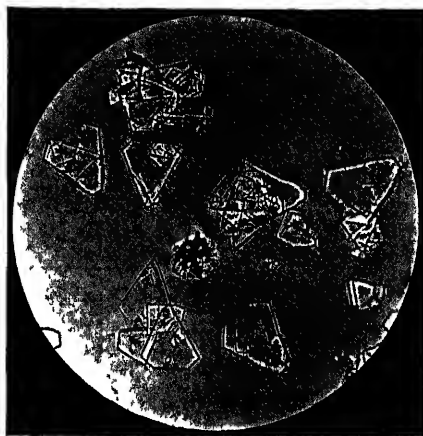
FIG. 1. Lanthionine from wool   (Photomicrograph by Mr. G. L. Keenan.)

cating the presence of amino groups in the α position to the carboxyl. All the nitrogen was found to be in the amino form. Titration with NaOH showed the presence of two carboxyl groups, and corresponded to an equivalent weight of 101.3.

Iodine Absorption Number—One of the characteristic properties of thio ethers is the capacity to absorb 2 atoms of halogen under certain conditions. It was, therefore, considered of interest to determine the iodine absorption value of the new amino acid. This determination was carried out according to the Hanus method (4). For comparison, a similar determination was made on methionine. The results are given in Table I. The new com-

pound was found to absorb the theoretical 2 atoms of iodine in 10 minutes, while methionine, on the other hand, required 40 minutes. The absorption values for both compounds remained constant thereafter for several hours. Assuming that the constant absorption value found for the new compound represented 2 atoms of iodine, a calculated molecular weight of 209 was derived. This is in fairly close agreement with that corresponding to the empirical formula.

Optical Rotation—A preliminary test on 5 mg. of the compound showed that it was optically inactive. A determination was then

TABLE I

Iodine Absorption Values for Thio Ether Amino Acid (I), and for Methionine

Time	Thio ether (I) (0 2106 gm.)		Methionine (0.20824 gm.)	
	Iodine absorbed	Calculated for 2 atoms	Iodine absorbed	Calculated for 2 atoms
min.	*mg.*	*per cent*	*mg*	*per cent*
5			298	84
10	255	100	312	88
20	255	100	327	92
30	269	105	341	96
40			355	100
50	255	100	355	100
hrs.				
1	255	100	355	100
18	255	100	355	100
24	326	127		

made at 20° on 60.606 mg. of the substance dissolved in 10 cc. of normal HCl. No optical rotation was observed.

Dibenzoyl Derivative—0.1 gm. of the compound was dissolved in 5 cc. of N NaOH in 10 cc. of water. 1 cc. of benzoyl chloride was added in small portions with vigorous shaking. The mixture was maintained at a slightly alkaline reaction by addition of NaOH. After the odor of benzoyl chloride had disappeared, 20 cc. of water were added and the solution was acidified with HCl. The voluminous precipitate was filtered off and extracted with benzene in order to remove benzoic acid. The residue was then recrystallized from 50 per cent alcohol. The dibenzoyl derivative

separated in the form of long prisms, melting at 205–206° (uncorrected).

Analysis—

$C_{20}H_{20}O_6N_2S$.　　　Calculated. N 6.75
5.619 mg. substance.　Found.　　" 6.80
7.038 "　　"　　　　"　　　" 6.60

Properties of the Compound—The compound is difficultly soluble in water and insoluble in alcohol, ether, chloroform, and acetone. It is readily soluble in dilute mineral acids and alkalies. It is optically inactive. When heated, it softens at 270°, and decomposes at about 304°. It is stable toward alkalies. When heated with 40 per cent NaOH in the presence of lead, there is no lead darkening. When dissolved in dilute ammonia and the solution allowed to evaporate slowly at room temperature, the thio ether separates in the form of six-sided plates having a triangle-like appearance (Fig. 1). We are indebted to Mr. G. L. Keenan for the following characterization of the crystals.

When examined in ordinary light, it consists of six-sided plates, more or less wedge-shaped in habit. In parallel polarized light (crossed nicols) the plates extinguish sharply and no interference figures were observed in convergent polarized light (crossed nicols). On account of the absence of interference figures, a variation of the statistical method was used in determining the refractive indices, selecting plates showing maximum double refraction in determining the lowest and highest indices of refraction. The refractive indices as determined by the Becke line method are $n_\alpha = 1.605$, $n_\gamma = 1.647$, both ±0.002.

The composition, chemical behavior, and properties of the new amino acid pointed very definitely to a thio ether amino acid having the structure,

$$HOOC—CH—CH_2—S—CH_2—CH—COOH$$
$$\quad\quad\quad NH_2 \quad\quad\quad\quad\quad\quad NH_2$$
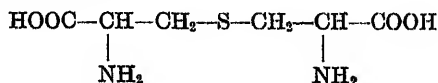
I. β-Amino-β-carboxyethyl sulfide

That the above represents the correct structure has now been definitely established by synthesis by du Vigneaud and Brown (5), an account of which is described in the following article.

A microscopical examination by Mr. Keenan of the synthetic compound, kindly furnished us by Dr. du Vigneaud, showed

that the crystal habit and the optical-crystallographic data were identical with those of the compound isolated from wool.

Because this amino acid has been first isolated from wool, and contains sulfur, it has been named *lanthionine.*

DISCUSSION

Until recently the number of sulfur-containing amino acids which have been isolated from naturally occurring material included only the two well known hydrolytic products of proteins; namely, cystine and methionine. Recently, the isolation of two other amino acids containing sulfur has been reported, (a) the thio ether of Küster and Irion (2), $COOH—CH(NH_2)—CH_2—CH_2—S—CH_2—CH(NH_2)—COOH$, which was obtained by the action of sodium sulfide on wool, and (b) djenkolic acid, $COOH—CH(NH_2)—CH_2—S—CH_2—S—CH_2—CH(NH_2)—COOH$, isolated from the djenkol bean by van Veen and Hyman (6) in 1933, and synthesized by du Vigneaud and Patterson (7) in 1936.

Goddard and Michaelis (8) have shown that keratins, such as wool and feathers, are dissolved in a few hours at room temperature at alkaline reaction with a solution of Na_2S, KCN, or thioglycolic acid. Products prepared from the solutions behave as true proteins, and not as products of hydrolysis. They are soluble in dilute acids and alkalies, and are digestible by pepsin or trypsin. The effects are produced chiefly by the splitting of disulfide bonds.

The action of dilute Na_2CO_3 solution on wool, however, is quite different from that produced by the reagents used by Goddard and Michaelis, although both involve a splitting of disulfide bonds. When wool is heated for 1 hour with dilute Na_2CO_3, very little is apparently dissolved and the wool retains its fibrous appearance. Nevertheless, marked changes occur. The Na_2CO_3-treated wool is no longer soluble in sodium sulfide, and unlike the product obtained by treating wool with sodium sulfide, it is not digestible by pepsin or trypsin. Although containing about three-fourths of the sulfur of the untreated wool, it yields no cystine when hydrolyzed with HCl, whereas both optically active and inactive cystines were isolated from the sodium sulfide product.

Evidently preliminary treatment of a protein with reagents

such as Na_2S and dilute Na_2CO_3, under favorable conditions, can bring about structural changes in the molecule so that it will give on subsequent hydrolysis products that are very different from those yielded by direct hydrolysis of the original protein. This consideration suggests the interesting possibility that other mild treatments, chemical or physical, might determine to some extent the kind of amino acids a protein will yield on hydrolysis.

The new thio ether here described is of interest from a number of different angles. It is the first instance of the isolation of a symmetrical thio ether amino acid obtained from the hydrolytic products of a protein. The question whether other proteins than wool, or the fibrous keratins, will yield thio ethers under similar treatment remains an interesting possibility. In this connection it is suggestive that van Veen and Hyman isolated djenkolic acid by prolonged treatment of the beans with barium hydroxide at 30°. The mechanism of the reaction between wool and dilute sodium carbonate, whereby the disulfide bonds of cystine are split with the formation of a grouping which on hydrolysis with acid yields thio ether amino acids, opens up a field of investigation which may be of great value in elucidating problems relating to protein structure. The structural relationship of the thio ether amino acid to cystine and methionine suggests interesting questions in connection with sulfur metabolism.

The thio ether may also be of particular interest to the organic chemist as a basic material for use in the synthesis of new organic compounds.

Feeding experiments are in progress in order to find out whether the thio ether amino acid which we have isolated from wool can replace cystine or methionine in the diet with respect to their growth-promoting properties.

A study of hair and feathers is in progress in order to see whether these keratins will also yield thio ethers.

SUMMARY

A new thio ether diamino acid, HOOC—CH(NH₂)—CH₂—S—
CH₂—CH(NH₂)—COOH, β-amino-β-carboxyethyl sulfide, has
been isolated by acid hydrolysis of wool that had been boiled
for 1 hour with 2 per cent sodium carbonate solution. It crystal-
lizes in plates, which are difficultly soluble in water and readily
soluble in dilute acids and alkalies. It is optically inactive, and
decomposes at about 304°. Because it was first isolated from
wool, and contains sulfur, the name *lanthionine* has been given
to this amino acid.

BIBLIOGRAPHY

1. Horn, M. J., and Jones, D B , *J. Am Chem. Soc.*, **62**, 234 (1940).
2. Küster, W., and Irion, W., *Z. physiol. Chem.*, **184**, 225 (1929).
3. Hoffman, W. F., *J. Biol. Chem* , **65**, 251 (1925).
4. Official and tentative methods of analysis of the Association of Official
   Agricultural Chemists, 4th edition, 410 (1935).
5. du Vigneaud, V., and Brown, G. B , *J. Biol. Chem.*, **138**, 151 (1941).
6. van Veen, A. G., and Hyman, A. J., *Geneesk. Tijdschr. Nederl.-Indie*,
   73, 991 (1933)
7. du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem* , **114**, 533 (1936).
8. Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, **106**, 605 (1934)

THE SYNTHESIS OF THE NEW SULFUR-CONTAINING AMINO ACID (LANTHIONINE) ISOLATED FROM SODIUM CARBONATE-TREATED WOOL

By VINCENT du VIGNEAUD and GEORGE BOSWORTH BROWN

(From the Department of Biochemistry, Cornell University Medical College, New York City)

A new sulfur-containing amino acid, obtained from wool which had been treated with sodium carbonate, is described in the preceding paper by Horn, Jones, and Ringel (1). On the basis of their analytical studies they suggested the following structure, and proposed the name lanthionine for the compound.

$$\underset{\beta\text{-Amino-}\beta\text{-carboxyethyl sulfide}}{\overset{\displaystyle \text{HOOC--CH--CH}_2\text{--S--CH}_2\text{--CH--COOH}}{\overset{\displaystyle |\qquad\qquad\qquad\quad |}{\overset{\displaystyle \text{NH}_2\qquad\qquad\quad\text{NH}_2}{}}}$$
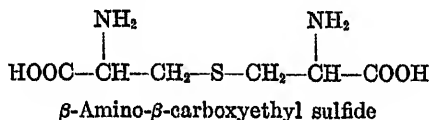
By arrangement with the above authors the preparation of this amino acid was undertaken by us to offer synthetic proof of the proposed structure. The synthesis was accomplished by the application of the procedure which we had already developed for the synthesis of S-(β-amino-β-carboxyethyl)-homocysteine (2). *l*-Cysteine was allowed to react with methyl *dl*-α-amino-β-chloropropionate hydrochloride in strongly alkaline solution. A mixture of crystalline compounds was isolated from the reaction product. The triangle-like plates characteristic of the compound described by Horn, Jones, and Ringel were observed, along with typical hexagonal crystals of *l*-cystine which had resulted from the oxidation of the unchanged cysteine. In addition to these two forms a goodly amount of tetragonal plates was also present. By careful fractionation a sample consisting exclusively of the triangle-like plates was obtained. This crystalline compound was compared with the sample of the isolated amino acid which had been kindly furnished us by Dr. Jones.

The chemical and physical properties of the synthetic and isolated samples were the same. The dicarbobenzoxy and dibenzoyl derivatives were prepared and the comparison of their properties afforded confirmation of the identity of the synthetic and isolated products.

Both the synthetic and the isolated materials were found to be optically inactive. From the fact that the synthetic compound resulted from the combination of a derivative of dl-α-amino-β-chloropropionic acid and l-cysteine it was to be expected that, unless racemization took place, only two diastereoisomeric forms, *meso* and l, would result. Since there is no reason to suspect that extensive racemization had occurred, it is to be assumed that the optically inactive triangle-like crystals represent the *meso* modification.

EXPERIMENTAL

β-Amino-β-Carboxyethyl Sulfide—30 gm. of cystine $[\alpha]_D^{20} = -213°$ were dissolved in 500 cc. of liquid NH_3 and metallic Na was added until the appearance of a blue color indicated that the reduction was complete. The NH_3 was allowed to evaporate and the flask was evacuated for 2 hours to remove as much NH_3 as possible. 110 gm. of 33 per cent KOH were added and the mixture was warmed to 45°. With continuous stirring, 20 gm. of the methyl α-amino-β-chloropropionate hydrochloride (2) were added over a period of 1 hour, and the mixture was stirred for an additional 2 hours. The product was diluted with 2 volumes of water and, after it had been cooled in an ice bath, it was neutralized with concentrated HI. The solution was then made alkaline to litmus with NH_4OH; a drop of $FeCl_3$ was added, and the solution was gently aerated to oxidize the cysteine present. It was then concentrated to a volume of 250 cc., was brought to approximately pH 6, and 2 volumes of alcohol were added. The precipitate, which amounted to 35 gm., was dissolved in 200 cc. of water by the use of a minimum quantity of dilute HCl, and 2 N NH_4OH was added very slowly, with stirring, over a period of several hours. The crystallization was followed by microscopic examination. While the solution was still distinctly acid to Congo red, about 12 gm. of l-cystine were removed by filtration.

The next crop (Fraction B, 9 gm.), obtained at a pH of 5 to 6, consisted of about 50 per cent of the typical hexagons of *l*-cystine mixed with triangle-like and tetragonal plates. A trace of elongated hexagons was present. The last crop (6 gm.), obtained at a pH of 6 to 7, contained all of the above forms, as well as indefinite crystalline forms.

One-half of Fraction B was dissolved in 5 cc. of concentrated HCl and the cystine hydrochloride which formed was separated. The filtrate was diluted and slowly neutralized with dilute NH_4OH. The product which precipitated still contained some cystine. It was dissolved in 20 cc. of water by the addition of a minimum amount of HCl and fractionally precipitated by slow neutralization with 2 N NH_4OH. The formation of crystals was followed by frequent examination with the microscope. A total of twelve fractions was separated and the middle fractions, which were preponderantly triangle-like plates, were refractionated. The third refractionation yielded 0.5 gm. of a sample which was exclusively the triangle-like plates. The other half of Fraction B was put through a similar fractionation and an additional 0.5 gm. of the same product was obtained. The compound decomposed slowly above 270° and completely at 308°. It had the following composition.

$C_6H_{12}O_4N_2S$.   Calculated.   C 34.60, H 5.81, N 13.45, S 15.40
                     Found.   " 34.49, " 6.13, " 13.41, " 15.50

A detailed optical-crystallographic comparison of the synthetic with the isolated sample is reported in the paper by Horn, Jones, and Ringel (1).

A test for optical activity was made on a 1 per cent solution in 1 N HCl and on a 1 per cent solution in concentrated HCl. No rotation was observed for either the synthetic or isolated material.

To make certain that the compound did not arise from the action of the alkali on the cysteine itself a control experiment was run in which cysteine was treated with 33 per cent KOH at 45°. No indication of the formation of lanthionine was detected.

N,N'-Dibenzoyl-β-Amino-β-Carboxyethyl Sulfide—20 mg. of the β-amino-β-carboxyethyl sulfide were dissolved in 2 cc. of 0.5 N NaOH and 0.1 cc. of benzoyl chloride was added with mechanical stirring. The mixture was acidified with 1 cc. of N HCl. The

precipitate, after extraction with benzene, was recrystallized from 50 per cent alcohol. The melting point of the derivative was 210–211° (corrected) which agrees with that determined for the dibenzoyl derivative of the isolated compound (1). A mixture of this compound with the corresponding derivative of the isolated amino acid showed no depression of the melting point.

N,N'-Dicarbobenzoxy-β-Amino-β-Carboxyethyl Sulfide—20 mg. of the β-amino-β-carboxyethyl sulfide were dissolved in 0.5 cc. of N NaOH and 0.1 cc. of 90 per cent carbobenzoxy chloride was added and the mixture stirred until the odor of carbobenzoxy chloride disappeared. The solution was diluted to 20 cc., was extracted with ether, and was slowly acidified with HCl. The dicarbobenzoxy derivative which crystallized on standing was collected and was recrystallized from 30 per cent alcohol. The compound crystallized as fine needles. It melted at 138–140° (corrected) and had the following composition.

$C_{22}H_{24}O_8N_2S$.   Calculated, N 5.88; found, N 5.68

The dicarbobenzoxy derivative of the isolated compound was also prepared and found to possess the same properties as that of the synthetic compound. It also melted at the same temperature and a mixture of equal parts of this derivative with that prepared from the synthetic compound showed no depression of the melting point.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

BIBLIOGRAPHY

1. Horn, M. J., Jones, D. B., and Ringel, S. J., *J. Biol. Chem.*, **138**, 141 (1941).
2. Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **137**, 611 (1941).

RATE OF FORMATION AND DISAPPEARANCE OF BODY CREATINE IN NORMAL ANIMALS*

By KONRAD BLOCH, RUDOLF SCHOENHEIMER, AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

It has been recently shown (1) that most physiological compounds so far investigated with isotopes (proteins, amino acids, fatty acids, cholesterol) are continuously ruptured and rebuilt at a rapid rate in normal animals even when their weight is constant. These biochemical reactions, which leave unchanged the composition of the animal tissues, can conveniently be measured with the aid of isotopes, and have been termed molecular regenerations.

Preliminary to a study with isotopes of the biological precursors of creatine it was found necessary to determine whether or not, in addition to the continuous replacement of excreted material (creatinine), there also occurs in normal animals a molecular regeneration of body creatine; *i.e.*, an automatic degradation together with a corresponding synthesis. A reaction of this kind would not have become apparent by any investigation of the creatine balance, as the total amount of the substance would remain unchanged.

There are two isotope methods available for the investigation of the rate of regeneration of a biological compound in normal animals, (a) the determination of the rate of its appearance, and (b) of its disappearance. For convenience we have determined the rate of disappearance of isotopic creatine from the tissues of adult rats kept on a creatine-free diet.

As reported previously (2), the addition of isotopic creatine in small amounts to the diet leads to its deposition, resulting in an

inseparable mixture of the preformed and the newly deposited material. The body creatine thereby acquires an isotopic marker. The creatinine excreted after discontinuance of creatine feeding had an isotope concentration exactly that of the body creatine. Hence the creatinine excreted on a creatine-free diet is to be regarded, on the basis of these results, as representative of the total body creatine.

Rate of Replacement of Body Creatine on Creatine-Free Diet

As in our earlier experiments, three adult rats were given in addition to their normal stock diet 53 mg. daily per kilo of body
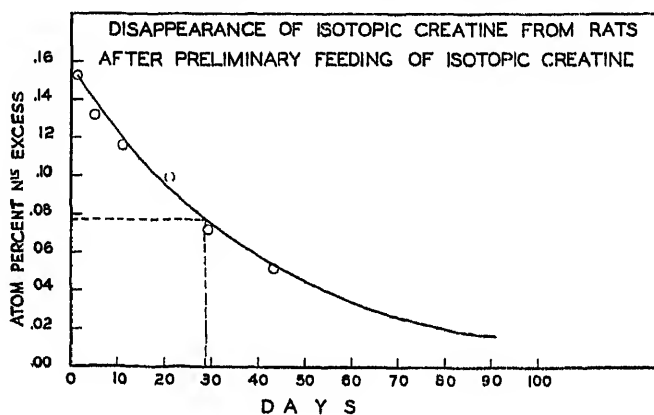
FIG. 1

weight of isotopic creatine for 6 days. The animals were then kept for 43 days on the same creatine-free diet. At intervals samples of creatinine were isolated from the urine. The isotope content of each sample was determined; this, according to the previous investigation, must have been the same as that of the body creatine at the time the creatinine was excreted. The isotope concentration in the compound decreased steadily with time (see Fig. 1).

The continuous decrease of the isotope concentration in the urinary creatinine indicates the continuous disappearance of creatine originally present in the tissues. An amount of creatine corresponding to that lost must have been newly formed. *The curve (Fig. 1) thus is a measure of the rate of disappearance and*

simultaneous synthesis of body creatine. This rate is rather slow. Within 24 hours the original isotope content decreases by about 2 per cent; *i.e.,* an amount of creatine is formed daily which corresponds to about 2 per cent of the total body creatine.

The animal continuously loses creatine in the form of creatinine. We have determined by analysis of the urinary creatinine and body creatine what fraction of the body creatine was converted and excreted. In three experiments the rats excreted daily an amount of creatinine corresponding to 1.9, 2.1, and 2.0 per cent of that of their total body creatine. These amounts correspond approximately to those formed by synthesis. *The close agreement between the values for daily excretion and synthesis excludes the occurrence of any major metabolic pathway other than that of creatinine formation and excretion.* This is further indicated by the finding that the urea excreted by the animals given isotopic creatine contained no, and the urinary ammonia hardly any, significant concentration of marked nitrogen. As the amounts of isotopic creatine given are very small compared to that of urinary urea, a small degradation might have escaped detection. Our findings, obtained with normal animals on a creatine-free stock diet, do not exclude the possibility that even extensive creatine degradation may occur when the compound is administered in large quantities. We have, however, not carried out experiments of this kind with isotopes, as we were primarily interested in the normal metabolism of creatine.

The isotope concentration in the creatine of our animals had decreased to half its original value within about 29 days. This is therefore the time during which half[1] of the creatine molecules of rats on a creatine-free diet are replaced by newly formed ones.

Biological Stability of Amidine Group in Creatine

The experiments described above were carried out with a creatine preparation (I), the marked nitrogen of which was that direct-

---

[1] The curve (Table I) shows again the advisability of determining the rate of such replacement reactions by their *half time* value rather than by the time when the reaction is completed. It will take an infinite time until the last molecule of isotopic creatine has disappeared. In the present experiment it would take over 100 days for the isotope concentration in the body creatine to be so low as to escape analytical detection. The determination of the end of the reaction becomes thus merely a determination of the error of the analytical method.

ly attached to the carbon chain, the amidine group (NH_2C=NH)

containing only normal nitrogen. If this group or its nitrogen had been detached and replaced in the living body by nitrogen from other sources, such a process would not have been detected; it would have resulted merely in the replacement of normal nitrogen by normal nitrogen and would not have affected the total amount of creatine or its isotope content. Such a replacement of amidine nitrogen has been found to occur continuously in normal animals with the arginine of the proteins (3). Its amidine group is continuously split off, and the ornithine liberated recombines with nitrogen from other sources to regenerate arginine.

For the investigation of the biological stability of the amidine group it was necessary to employ a creatine preparation with isotopes in the amidine group as well as in the remaining part of the molecule. We have prepared a sample of creatine (II) in which all 3 nitrogen atoms were labeled. The material was ob-

$$
\begin{array}{cc}
NH_2 & N^*H_2 \\
| & | \\
C{=}NH & C{=}N^*H \\
| & | \\
N^*CH_3 & N^*CH_3 \\
| & | \\
CH_2COOH & CH_2COOH \\
(I) & (II)
\end{array}
$$
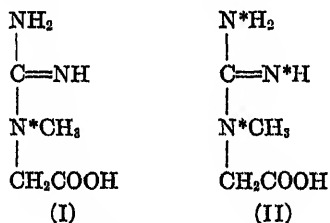
The asterisks designate marked nitrogen atoms.

tained by condensing isotopic cyanamide with isotopic sarcosine. The cyanamide sample was prepared by the reaction of isotopic ammonia with normal, non-isotopic cyanogen bromide.

$$2N^*H_3 + BrC{\equiv}N \rightarrow N^*H_4Br + N{\equiv}C{-}N^*H_2$$

$$
N{\equiv}C{-}N^*H_2 +
\begin{array}{c}
HN^*CH_3 \\
| \\
CH_2COOH
\end{array}
\rightarrow
\begin{array}{c}
N^*H_2 \\
| \\
C{=}N^*H \\
| \\
N^*CH_3 \\
| \\
CH_2COOH
\end{array}
$$
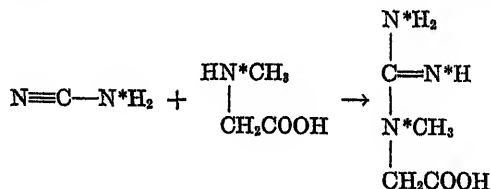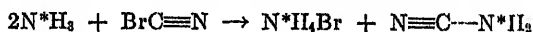
According to the mode of preparation, 1 of the nitrogen atoms of the amidine group should be isotopic, while the other should be

normal. α,α-Disubstituted guanidines, however, are assumed to resonate between the structures (A) and (B) (4).   The 2 nitrogen

$$
\begin{array}{cc}
\overset{\cdot\cdot}{NH_2}\diagdown\quad\diagup\overset{+}{NH_2} & \overset{+}{NH_2}\diagdown\quad\diagup\overset{\cdot\cdot}{NH_2} \\
C & C \\
| & | \\
NR' & NR' \\
R'' & R'' \\
(A) & (B)
\end{array}
$$
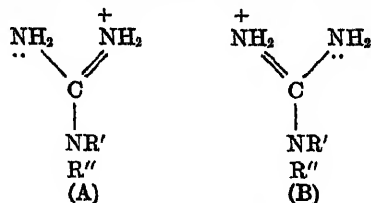
atoms of the amidine group should accordingly be functionally equal and indistinguishable with regard to their isotope content. For our considerations we may assume that each of them contains one-half of the isotope concentration originally present in the ammonia.[2]   In our formula we have therefore designated both nitrogen atoms as isotopic.

On treatment of creatine with boiling aqueous barium hydroxide the nitrogen of the amidine group is liberated as ammonia (6, 7), while the remaining nitrogen can be recovered from the solution as sarcosine (in the form of its β-naphthalenesulfonyl derivative).

$$
\begin{array}{ccc}
NH_2 & & \\
| & & 2NH_3 + CO_2 \\
C{=}NH & \xrightarrow{Ba(OH)_2} & + \\
| & & HNCH_3 \\
NCH_3 & & | \\
| & & CH_2COOH \\
CH_2COOH & &
\end{array}
$$
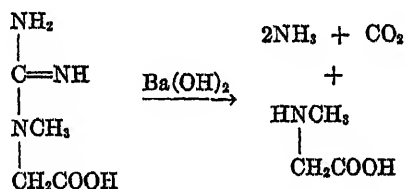
By subjecting to this procedure the synthetic creatine (II), or the compound isolated after its feeding, the concentration of the isotopes in each of the two groupings of the respective creatine sample can be ascertained.   The creatine molecule (II) thus contains two independent isotopic markers.   A closer investigation of this degradation also supports the concept of the equal function of the

[2] We have employed in our formulation of cyanamide the classical structure instead of the symmetric carbodiimide structure ($NH{=}C{=}NH$) proposed by Werner (5).   Tautomerism would also result in a statistical distribution of the marked nitrogen atoms.   The resonance of the amidine group of creatine renders, in our special case, the choice between the two cyanamide structures immaterial.

2 nitrogen atoms in the amidine group. Consecutive samples of ammonia liberated from creatine (II) by treatment with barium hydroxide all had practically the same isotope concentration, indicating that the nitrogen atoms originating from the two distinct sources are indistinguishable.

If isotopic creatine is fed to an animal, it mixes with the normal creatine of the body, whereby the isotope concentration of the administered material is correspondingly lowered. If a sample of the isolated creatine is degraded with barium hydroxide, the two independent isotope markers of creatine (II) should be equally diluted; *i.e.*, the ratio of the isotope concentrations (in atom per

TABLE I

Biological Stability of Creatine Molecule

Rats were given creatine with isotopic nitrogen in the amidine as well as in the sarcosine group.

	N^{15} content in			$\dfrac{N^{15} \text{ in amidine}}{N^{15} \text{ in sarcosine}}$
	Total compound	Amidine group	Sarcosine group	
	atom per cent excess	*atom per cent excess*	*atom per cent excess*	
Material administered.....	5.03	5.25	4.50	1.17
Urinary creatinine excreted during whole feeding period ..	0.227	0.241	0.219	1.10
Muscle creatine 18 days after last feeding of isotopic creatine..	0.284	0.287	0.259	1.11

cent) in both groupings, amidine and sarcosine, should be the same as in the compound administered. If, however, in addition to the biological admixture, the amidine nitrogen were replaced by normal nitrogen (as was found to occur with arginine), the ratio must change.

Three fully grown rats were given 53 mg. of isotopic creatine (II) per kilo of body weight daily for 6 days; the animals were then kept for the following 18 days on a creatine-free diet, and then killed. Creatine was isolated from the muscles and creatinine from the urine. After the isotope in the total nitrogen of all three samples had been analyzed, each sample was degraded with barium hydroxide. The results of the isotope analyses are given

in Table I. The ratio of the nitrogen isotope concentrations in the two fractions of the synthetic material was 1.17. The same ratio, within the limit of error, was found in the urinary creatinine, as well as in the muscle creatine 18 days after the last feeding of the isotopic compound. *The results show definitely that the amidine group of creatine had remained attached to the sarcosine part and had not been replaced.* There is thus no analogy to the metabolic activity of the amidine group in arginine.

The two experimental series taken together illustrate the high biological resistance of the creatine molecule. Creatine is known to be reversibly phosphorylated and dephosphorylated at a rapid rate, but in animals on a creatine-free diet it seems to undergo only one other metabolic reaction; namely, the irreversible (2) conversion to creatinine. It thus differs in its metabolic aspect from all other biological compounds so far investigated with isotopes. It is continuously synthesized but not significantly degraded. Once formed the creatine molecule proper is not involved in any biochemical reaction in which linkages between carbon and carbon or carbon and nitrogen are broken.

EXPERIMENTAL

Synthesis of Isotopic Creatine Samples

Both isotopic creatine samples (I) and (II) were prepared by condensation of sarcosine with cyanamide in the presence of ammonia. The preparation of isotopic sarcosine and of creatine (I) has been described before (2). For the preparation of creatine (II) the same procedure was employed with isotopic cyanamide.

Isotopic Cyanamide—1.07 gm. of ammonium chloride containing 10.5 atom per cent N^{15} excess were treated in a closed system with sodium hydroxide and the ammonia liberated was carried over by a slow stream of nitrogen, through a drying tube filled with potassium hydroxide, into 15 cc. of absolute methanol, cooled by solid carbon dioxide in acetone. Any unabsorbed ammonia was collected in dilute sulfuric acid. 1.06 gm. of freshly prepared non-isotopic cyanogen bromide in 20 cc. of absolute ether were added to the ammoniacal methanol, and the mixture was kept for 12 hours at room temperature. The ammonium bromide formed was filtered off and washed with absolute ether, and the combined filtrate and washings were brought to dryness *in vacuo*. The

residue was extracted exhaustively with dry ether and the ether removed by a stream of dry nitrogen. The crystalline cyanamide (0.40 gm.) was not purified further.

Isotopic Creatine (II)—0.445 gm. of sarcosine containing 4.50 atom per cent N^{15} excess was dissolved in 2.0 cc. of water containing 2 drops of concentrated non-isotopic ammonia, and the above cyanamide in 3 cc. of water was added. The solution was kept for 48 hours at room temperature. The creatine hydrate was filtered off and recrystallized from a small volume of water. Yield 0.445 gm., corresponding to a yield of 30 per cent calculated on the isotopic ammonia employed for the preparation of cyanamide and 60 per cent calculated on the sarcosine. The compound contained 28.3 per cent N (calculated 28.2).

The creatine so prepared contained 1 atom of normal nitrogen, 1 atom containing 10.50, and 1 containing 4.50 atom per cent excess. The total molecule should contain $(0 + 10.50 + 4.50)/3 = 5.00$ atom per cent excess. The value found was 5.03 atom per cent, which is within the limits of error.

For the condensation of cyanamide with sarcosine normal ammonia had been added as a catalyst. The agreement of the calculated and found isotope values in the creatine shows that the nitrogen of the ammonia had not exchanged with that of the cyanamide. The stability of the creatine nitrogen has been demonstrated before (2).

Isolation and Degradation of Creatine Samples—Samples of creatine from muscles and creatinine from urine were isolated as previously described (2). For separate analysis of the amidine and the sarcosine groups the samples of potassium creatinine picrate were suspended in concentrated hydrochloric acid and picric acid removed by continuous extraction with ether. The colorless aqueous solutions were brought to dryness *in vacuo* and the residue boiled for 24 hours in a solution containing 10 per cent barium hydroxide. The ammonia liberated was carried by a slow stream of nitrogen into wash bottles containing dilute sulfuric acid.

Barium was removed with sulfuric acid, and the filtrates brought to a small volume and treated in a test-tube by stirring with alkali and β-naphthalenesulfonyl chloride in the usual fashion. The naphthalenesulfonyl sarcosine samples obtained after acidification

were recrystallized from water until the melting point was 172–173°.   Nitrogen (Kjeldahl) found 5.1, 5.1; calculated 5.0.

The samples of ammonia and of naphthalenesulfonyl sarcosine were analyzed separately for isotope.

The purity of all samples was determined (a) by analysis of the creatinine nitrogen after removal of picric acid with ether, and (b) by colorimetric creatinine determination of the picrates.

Fractional Degradation of Isotopic Creatine—4.4 mg. of creatine (II) and 91.1 mg. of ordinary creatine were refluxed in 10 per cent barium hydroxide solution and the ammonia liberated was carried by a continuous stream of nitrogen into wash bottles containing dilute sulfuric acid.   After various time intervals the traps were removed and the amount of total ammonia and its isotope content determined.   The values are given in Table II.

TABLE II

Fractional Degradation of Isotopic Creatine

Trap No.	Time	Ammonia liberated	
		mg.	*atom per cent* N^{15} *excess*
1	10 min.	0.11	
2	70 "	4.27	0.231
3	3 hrs.	2.37	0.234
4	6 "	4.09	0.225
5	24 "	6.51	0.238

The amount of ammonia nitrogen liberated, 17.35 mg., corresponded to 97 per cent of the calculated amount (17.82 mg.).

Biological Experiments

Determination of Body Creatine and Urinary Creatinine—Urinary creatinine was determined quantitatively according to Folin (8). For the determination of body creatine, the total carcass was continuously extracted for 8 hours with boiling 95 per cent ethanol. The alcoholic solutions were concentrated *in vacuo* and distributed between ether and water.   The aqueous layer was acidified with hydrochloric acid and creatine converted into creatinine by refluxing for 2 hours.   After concentration *in vacuo* and treatment with lead acetate, creatinine was determined colorimetrically in aliquots.   The values are given in Table III.

Rate of Replacement of Body Creatine on Creatine-Free Diet—

Three male rats of 1120 gm. combined weight were kept in metabolism cages. To the stock diet, containing casein 15, cornstarch 68, yeast 5, salt mixture (Osborne and Mendel (9)) 4, cod liver oil 2, and Wesson oil 6 per cent, were added 53 mg. of creatine (I) (calculated for anhydrous creatine) per kilo of body weight daily for 6 days. This creatine, with the isotope marker in the sarcosine part of the molecule only, contained 1.5 atom per cent N^{15} excess. After discontinuance of the creatine addition the animals were kept on the creatine-free stock diet for 43 more days.

TABLE III

Amount of Creatinine Excreted Relative to Body Creatine

Experiment No.	Weight of animals	Total body creatine	Urinary creatinine per day calculated as creatine	Fraction of body creatine excreted daily
	gm.	*mg.*	*mg.*	*per cent*
1	627	1160	21.4	1.9
2	606	1025	21.0	2.1
3	601	1206	26 4	2.0

TABLE IV

Decrease of Isotope in Creatine

	Days after last creatine feeding	N^{15} excess in material isolated
		atom per cent
Urinary creatinine	1	• 0.153
	5	0.132
	11	0.116
	21	0.099
	29	0.072
Muscle creatine	43	0.052

48 hour specimens of pooled urine were collected at intervals for isolation of creatinine. At the end of the period, the animals were killed and muscle creatine was isolated. The analytical values are given in Table IV.

Biological Stability of Creatine Molecule—The experimental conditions were similar. Three male rats of 999 gm. combined weight obtained an addition of 53 mg. of creatine (II) (calculated as anhydrous creatine) per kilo of body weight daily for 6 days, and were then kept on the stock diet without creatine addition.

Creatinine was isolated from the pooled urine samples collected during the period of creatine feeding, and muscle creatine was isolated after the animals were killed. A sample of urea isolated from the urine contained 0.003 ± 0.003, and ammonia 0.014 ± 0.003 atom per cent N^{15} excess. The isotope concentration in the nitrogen of the amidine and sarcosine groups, determined as described above, is given in Table I.

SUMMARY

1. The rate of creatine formation in normal rats on a creatine-free diet was investigated with the aid of two different isotopic creatine preparations: (I) contained N^{15} in the sarcosine part only and (II) contained N^{15} in the amidine as well as in the sarcosine part of the molecule. These compounds were synthesized from isotopic sarcosine with normal and isotopic cyanamide respectively.

2. Both compounds were added, in a preliminary period, to the stock diet of rats. The animals were subsequently kept on a creatine-free diet and creatinine was isolated from the urine at intervals. From the decrease of isotope concentration it was concluded that the amount of creatine synthesized daily corresponds to about 2 per cent of the total creatine of the animal tissues. This was found to be about the same quantity as is daily excreted as creatinine. Within 29 days half of the creatine molecules in adult rats on a creatine-free diet are replaced by new molecules.

3. By employing creatine (II) it was possible to follow separately the metabolic fate of both groupings of the creatine molecule, the amidine and the sarcosine parts, as the isotope in both groupings can be determined after degradation of the creatine. In contrast to the amidine moiety of arginine, that of creatine is not replaced during metabolism; the linkage between amidine and sarcosine groups remains intact.

4. The creatine of the tissue in a normal animal on a normal diet seems to undergo no major metabolic reaction involving disruption of C—C or C—N linkages.

BIBLIOGRAPHY

1. Schoenheimer, R., and Rittenberg, D., *Physiol. Rev.*, **20**, 218 (1940).
2. Bloch, K., and Schoenheimer, R., *J. Biol. Chem*, **131**, 111 (1939).

3. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
4. Pauling, L., The nature of the chemical bond, Ithaca, 2nd edition, 213 (1940).
5. Werner, E. A., *J. Chem. Soc.*, **107**, 726 (1917).
6. Liebig, J., *Ann. Chem. u. Pharm.*, **62**, 257 (1847).
7. Gaebler, O. H., *J. Biol. Chem.*, **69**, 613 (1926).
8. Folin, O., in Festschrift für O. Hammarsten, Upsala, iii, 1 (1906).
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).

THE BIOLOGICAL PRECURSORS OF CREATINE*

By KONRAD BLOCH AND RUDOLF SCHOENHEIMER

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

Most of the earlier investigations on the biological precursors of creatine and creatinine have employed balance experimentation on normal humans or animals. After administration of hypothetical precursors, the amounts of creatine (or creatinine) excreted were measured. None of these experiments has clearly revealed the precursors. The results were interpreted (3) as support of the view that creatine formation cannot be increased above the physiological needs of the cells by administration of amino acids.[1] There are two other types of experiments, however, that had definitely pointed to glycine and arginine as creatine precursors. Brand *et al.* (4) found that patients with muscle dystrophy (in contrast to normal humans or animals) excrete more creatine after administration of glycine, and Fisher and Wilhelmi (5) claimed that the addition of arginine to the perfusion fluid of rabbit hearts resulted in creatine formation. We abstain in the introduction from presenting the vast literature on creatine formation, which has recently been completely reviewed (3, 6, 7). We shall discuss some of these papers and also the recent revealing experiments of Borsook and Dubnoff on arginine (8), in conjunction with our own experiments.

The only substance which is known to result in extra creatine (or creatinine) formation after administration to normal animals or humans is guanidoacetic acid ((6) p. 224). While this sub-

[1] The later experimental results and theories of Beard and collaborators, which would require opposite conclusions, are discussed in an addendum to this paper.

stance is not a normal food constituent but has been found in small quantities in normal urine and tissues (9, 10), it may be regarded as an intermediate in creatine formation.   In the biological synthesis of creatine the following two steps thus appear to be established: (a) formation of guanidoacetic acid, and (b) methylation.

The formation of the total creatine molecule is a slow process; an amount corresponding to only about 2 per cent of the total creatine is synthesized per day by animals on a creatine-free diet (11).   Reaction (b), methylation, however, proceeds rapidly, as a considerable part of administered guanidoacetic acid is immediately converted into creatine.   The rate of creatine formation thus seems to be mainly restricted by the slow rate of reaction (a).   Once guanidoacetic acid is formed, in the normal metabolism, it should be easily methylated.

Reaction (b), methylation, has recently been elucidated.   Borsook and Dubnoff (12) have found that guanidoacetic acid was slowly converted into creatine by tissue slices, but that addition of methionine vastly increased the rate and extent of this process.   None of the other compounds investigated had the effect of methionine.   The findings were interpreted as indicating a shift of the methyl group from methionine to guanidoacetic acid to form creatine.   The occurrence of this reaction *in vivo* has been established conclusively by du Vigneaud *et al.* (13).   The administration of methionine containing deuterium in the methyl group resulted in the formation of deutero creatine (and creatinine).

The present paper is concerned with the source of nitrogen for creatine; *i.e.*, with the precursors of guanidoacetic acid.

We have tested twelve nitrogenous compounds, most of which are normal body constituents, as creatine precursors by adding a small amount of their isotopic analogues to the ordinary stock diet of normal rats.   We have thereby followed normal creatine formation.   The synthesis of some of the isotopic compounds employed has been described before; the methods of preparation of the others are given in this paper.[2]   Most experiments were carried out with adult male rats which, as has been shown previously, synthesize an amount corresponding to about 2 per cent of the total body

[2] The authors would like to express again their indebtedness to Dr. H. C. Urey for the valuable gift of isotopes, and especially for the high concentrations of N^{15} which were employed in the present study.

creatine per day. Only a few experiments were performed with immature, growing animals, which synthesized a larger fraction.[3] The addition of the isotopic compound extended over several days, after which the animals were killed and creatine was isolated from the bodies. In a few experiments creatinine was isolated from the urine. The isotope content of the isolated compounds was determined. As the rate of creatine formation is slow, the amount of isotopic creatine formed from the respective precursors during the short experimental period could not have been large, even if all of the newly formed creatine had been derived exclusively from the isotopic dietary addition. The isotopic amino acids tested, before being utilized, must have mixed with considerable quantities of their normal, non-isotopic analogues present in the animal tissues or in the casein of the diet, and dilution of the isotope concentration in the nitrogenous source for creatine must thereby have occurred.

Of the normal dietary and tissue components tested, *arginine and glycine were found to be exceptionally effective as creatine precursors*. The nitrogen of both compounds is thus utilized for creatine formation. They provide nitrogen, however, to different parts of the creatine molecule. Creatine has 3 nitrogen atoms. The presence of the isotope marker in the total creatine molecule did not by itself indicate the mechanism of its formation unless the position of the isotope in the creatine was located. For this purpose some of the isotopic creatine samples were degraded by boiling with aqueous barium hydroxide as described before (11), whereby the nitrogen of the amidine group was recovered as ammonia and the remaining nitrogen atom as sarcosine (I).

$$NH_2$$
$$|$$
$$C\!\!=\!\!NH \quad \xrightarrow{\text{Ba(OH)}_2} \quad 2NH_3 + CO_2 + \quad \begin{array}{l} HN\!\!-\!\!CH_3 \\ | \\ CH_2COOH \end{array}$$
$$|$$
$$N\!\!-\!\!CH_3$$
$$|$$
$$CH_2COOH$$

(I)

---
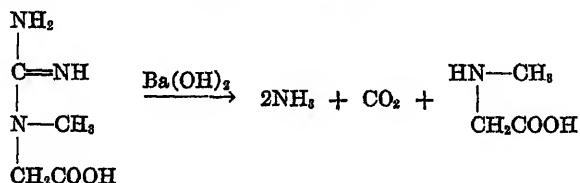
[3] This becomes apparent from those series of experiments listed in Table I in which the same amount of isotopic compound, *e.g.* glycine or guanidoacetic acid, was given to adult and immature rats. The isotopic content in the creatine of the bodies of the latter was about 3 times as high, indicating that the immature animals had synthesized about 3 times as much creatine; *i.e.*, about 6 per cent.

Separate isotope analysis of these two fractions revealed their origin. *The amidine nitrogen of creatine is derived from that of the corresponding group of arginine, and the nitrogen of the sarcosine group originates from glycine.*

Compounds Tested

Guanidoacetic Acid—The compound tested was prepared by the reaction of non-isotopic cyanamide with isotopic glycine (14). Following its addition in small amounts to the stock diet, both the creatine in the muscle and creatinine in the urine were found to have a high isotope content. The quantitative results of this and the following experiments (Table I) confirm the many reports that this substance is readily converted, *i.e.* methylated, to creatine. Its high effectiveness becomes apparent when the result is compared with an analogous experiment in which an equivalent amount of creatine (instead of guanidoacetic acid) was given (15). In both experiments the muscle creatine of the animals had about the same isotope concentration. Guanidoacetic acid when given in small amounts does not seem to be decomposed to an appreciable extent, as the urinary urea contained no marked nitrogen.

Hydantoic Acid and Methylhydantoic Acid—These two compounds were prepared by the action of normal potassium cyanate on isotopic glycine and sarcosine respectively (16, 17). Creatine isolated after their administration did not contain any marked nitrogen. The finding is in agreement with those of earlier investigators (18, 19), who failed to detect an increase of creatine formation or creatinine excretion after the feeding of these substances. In contrast to guanidoacetic acid, they cannot be considered as intermediates; their ureido group ($NH_2C{=}O$) is

$$\begin{array}{c} | \\ NR_2 \end{array}$$

not converted into the guanido ($NH_2C{=}NH$) group in the body.

$$\begin{array}{c} | \\ NR_2 \end{array}$$

On the other hand, analogous reactions with other substances may be possible: Klose and Almquist (20) have recently demonstrated the direct conversion in the chick of citrulline into arginine.

Ammonia—When given in moderate amounts (21) to adult

rats ammonia has a small effect as a source of creatine nitrogen. The isotope concentration in the compound isolated is just above the limit of error of the isotope analysis. However, by changing the experimental procedure creatine samples with a moderate isotope content can be obtained. This was done by employing immature rats and keeping them on a nitrogen-low basal diet.[4] The marked ammonia nitrogen represented 70 per cent of the total dietary nitrogen. Under these experimental conditions the utilization of other possible creatine precursors must have been relatively slight.

The isotopic creatine obtained from these animals was degraded. Most of the isotope was located in the amidine group, while the sarcosine contained only traces. The small amounts of nitrogen which had been utilized were thus mainly employed for the formation of the amidine group. It will be shown below that arginine had acted as an intermediate.

dl-Tyrosine, l(−)-Leucine, and dl-Glutamic Acid—Ammonia nitrogen has been shown to be utilized in a similar way, but to a lesser extent than amino acid nitrogen, for the reversible amination of amino acids (21). Amino acids which are not specific and direct precursors of creatine may thus be expected, like ammonia, to yield only a very small amount of their nitrogen for creatine formation. The creatine samples isolated after administration of the three amino acids had either no marked nitrogen or only traces. The compounds tested thus seem to be about as effective as ammonia. Creatine isolated after feeding *l*(−)-leucine was degraded, and most of the isotope was found to be present in the amidine group. The values, however, are so low that they include a considerable error.

Glycine—In contrast to ammonia and the three amino acids discussed, glycine is a highly effective creatine precursor. This is in agreement with the theory of Brand *et al.* (4). Its administration in small amounts results in the formation of creatine with a high isotope content. The isotope constitution of the sample is fundamentally different from that obtained after ammonia feeding. Most of the isotope is located in the sarcosine part and only traces in the amidine group. Glycine may thus be considered

---

[4] The experiment, carried out mainly for other purposes, has been discussed earlier (22).

as a specific precursor of the "sarcosine part" of the creatine molecule.

Arginine—Like creatine, arginine contains a guanido group and has been claimed by many investigators to be a precursor of creatine. The results obtained with balance experiments, however, were contradictory and, as stated above, did not give clear cut results. Recently Fisher and Wilhelmi (5) reported the perfusion of rabbit hearts with arginine to result in a quantitative conversion into creatine. This result led the authors to formulate a reaction whereby the total guanido group of arginine is utilized for that of creatine, and they revived the old theory of Czernecki (23), according to which guanidoacetic acid is formed by β oxidation of arginine (II). In later experiments by Davenport, Fisher, and Wilhelmi (24) it was found that the methyl group was derived

```
  NH2              NH2
   |                |
  C=NH             C=NH              NH2
   |                |                 |
  NH               NH               C=NH               NH2
   |                |                 |                  |
  CH2              CH2               NH                C=NH
   |                |                 |                  |
  CH2              CH2        -->    CH2        -->     NH
   |                |                 |                  |
  CH2              CH2               CH2                CH2
   |                |                 |                  |
 HC—NH2           C=O               CH2                COOH
   |                |                 |
  COOH     -->    COOH              COOH
```
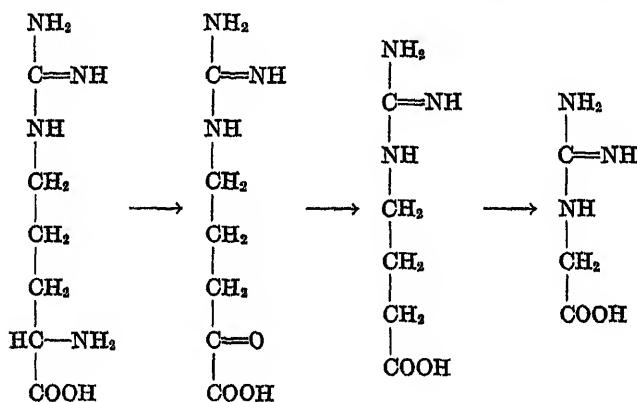
(II)

from glycolic acid, which might originate from glycine. This theory conforms neither with the results of Borsook and Dubnoff (12), and du Vigneaud and collaborators (13), on the rôle of methionine, nor with the present finding that glycine is employed for the formation of the "sarcosine part" of creatine.

In 1927 Bergmann and Zervas (25) performed *in vitro* experiments which suggested that the amidine group and not the total guanido group of arginine may be employed for creatine formation. Triacetylanhydroarginine when treated with water is split into urea and acetylanhydroornithine (acetylaminopiperidone). When

the reaction is carried out in a water-free medium in the presence of glycine ester or sarcosine ester, the amidine group is not liberated as urea but is transferred to the other component to form

$$CH_2COOC_2H_5$$
$$|$$
$$NH_2$$

$$+$$

$$HN—Ac$$
$$|$$
$$C=N—Ac$$
$$|$$
$$—N$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$HC—NHAc$$
$$|$$
$$—C=O$$

$$\longrightarrow$$

$$CH_2COOC_2H_5$$
$$|$$
$$NH$$
$$|$$
$$C=N—Ac$$
$$|$$
$$NHAc$$

$$+$$

$$—NH$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$HC—NHAc$$
$$|$$
$$—C=O$$

(III)
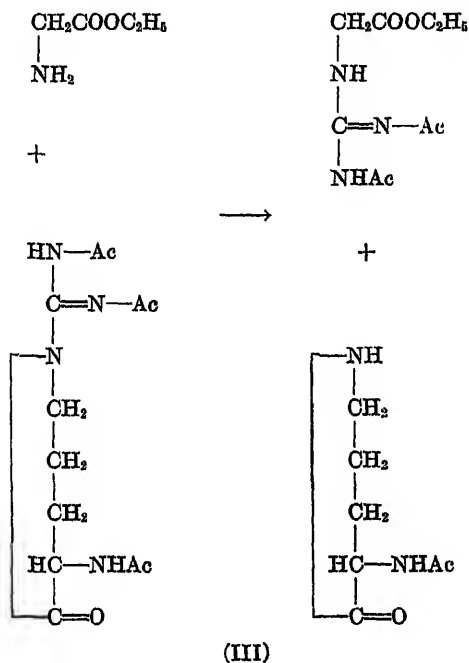
acetylguanidoacetic acid ester or acetylcreatine ester (III). In order to test the occurrence of the biological analogue to this highly suggestive chemical reaction it was necessary to synthesize and feed an arginine preparation, the amidine group of which contained isotope.

Synthesis of Isotopic Arginine—We have prepared this compound by replacing the amidine group in normal arginine by an isotopic amidine group. The α-amino group of $l(+)$-arginine was blocked by the preparation of α-toluenesulfonyl-$l(+)$-arginine (26), and the latter converted by boiling with aqueous baryta into α-toluenesulfoornithine (IV). This treatment resulted in partial racemization. As the two components were not easy to separate, resolution was postponed until a later stage of the syn-

Synthesis of Isotopic Arginine

$$2N^*H_3 + BrC\equiv N \rightarrow N^*H_2C\equiv N$$

$$N^*H_2C\equiv N + CH_3OH \rightarrow HN^*{=}C{-}N^*H_2$$

$$O{-}CH_3$$

NH$_2$ NH$_2$ N*H$_2$ N*H$_2$

C=NH   C=N*H   C=N*H

NH   NH$_2$ NH   NH

CH$_2$ CH$_2$ CH$_2$ CH$_2$

CH$_2$ Ba(OH)$_2$ CH$_2$ CH$_2$ Hydrolysis   CH$_2$

CH$_2$ CH$_2$ CH$_2$ CH$_2$

HC—NHSO$_2$R   HC—NHSO$_2$R   HC—NHSO$_2$R   HC—NH$_2$

COOH   COOH   COOH   COOH

(IV)

thesis. The partially racemic mixture was treated with isotopic methylisourea, prepared from isotopic cyanamide and methyl alcohol. The racemic toluenesulfoarginine could now be separated from the $l(+)$ compound because of the different solubilities of the components in water. The toluenesulfo group was removed by treatment with hot concentrated hydrochloric acid, and pure $l(+)$-arginine monohydrochloride[5] was obtained via the flavianate.

The addition of a small amount of this compound to the stock diet of rats resulted in the formation of creatine with isotope mainly in the amidine group. The result is thus qualitatively the same as that after feeding ammonia or α-amino acids (except glycine). Isotopic arginine, however, is by far more effective.

This result in conjunction with the others leads to the conclusion that the amidine group of arginine has been employed for creatine

[5] In our formula we have designated both nitrogen atoms of the amidine group as isotopic. This is in accordance with our formulation of isotopic creatine (11).

formation. The finding is well in accord with those obtained after feeding isotopic ammonia and α-amino acids. As already stated, administration of these compounds always results in the formation of arginine with isotope in its amidine group. If the amidine group of arginine is transferred in the process of creatine formation, any nitrogen-yielding isotopic substance must indirectly result in the presence of isotopic creatine.

Our experiments give no evidence for the utilization of the entire guanido group of arginine for creatine formation. It has been shown that the substituted nitrogen atom of creatine is derived from glycine. If the whole guanido group of arginine had been involved in creatine formation, the nitrogen from glycine should have reached the sarcosine part of the creatine molecule via the δ-amino group of ornithine. There is no evidence in the literature for the occurrence of such a process, which can also be excluded on the basis of former work with isotopes (27). When isotopic glycine was given, the nitrogen of the ornithine in the liver arginine contained only traces of marked nitrogen.

These observations on the rôle of arginine are in complete agreement with the recent results published in a preliminary note[6] by Borsook and Dubnoff (8). The authors had previously shown (12) the value of tissue slices for the investigation of creatine synthesis. They have now found kidney slices, in contrast to liver slices, rapidly to form guanidoacetic acid from arginine and glycine. The guanidoacetic acid was identified by isolation. These experiments thus also represent glycine and arginine as precursors of creatine. The finding was interpreted by the same chemical reaction; namely, by a shift of the amidine group.[7]

Histidine—The imidazole ring has been claimed, on the basis of balance experiments, to be a creatine precursor (28). We have not carried out direct conversion experiments by feeding isotopic histidine, but can offer indirect evidence against the theory that

[6] This note on the action of arginine in tissue slices appeared almost simultaneously with our preliminary note (2) on isotopic arginine.

[7] Borsook and Dubnoff (8) have proposed the term "transamidination." This term is coined according to "transamination," which is a reversible reaction. The work with isotopes has as yet given no indication that the shift of the amidine group is reversible. "Transamidination" might thus be misleading, and we shall refer to it as "amidine shift."

this compound is involved in creatine formation.   If the imidazole ring were the ultimate source or an intermediate, the animals that have formed isotopic creatine should also contain histidine with isotope in the imidazole ring.   Arginine and glycine, both of which are intermediates in creatine formation, were always found to contain isotope when isolated from such animals.

The imidazole ring of histidine does not take up nitrogen from ammonia or α-amino acids (29).   Rats do not seem to have the ability to form this group, a finding in agreement with the statement that histidine is an indispensable amino acid for rats (30). However, the same animals did form isotopic creatine.   The imidazole ring of histidine thus could not have been an intermediate.[8]

Urea—This is considered by Beard and Pizzolato (32) to be one of the most potent precursors of creatine.   Urea with a high isotope concentration (15 atom per cent excess) was prepared in good yields and without nitrogenous side products by the action of ammonia (N^{15}) with diphenyl carbonate[9] according to Hentschel (33).

The creatine isolated from the animals that had received this urea had only normal nitrogen.   Urea is not a creatine precursor.[10]

[8] Similar reasoning applies to the theory according to which uric acid (31) is a creatine precursor.   We have isolated allantoin from the urine of the rats (unpublished) that had received isotopic arginine.   The allantoin, which is the oxidation product of uric acid, had no appreciable amounts of isotope, while the creatine contained considerable quantities of marked nitrogen.

[9] The authors are indebted to Dr. DeWitt Stetten, Jr., for help with the synthesis.   When the procedure of Hentschel, worked out for 5 kilo lots, was repeated with 10 gm. samples of diphenyl carbonate, the reaction went extremely slowly and almost no urea was formed.   When a small amount of copper powder was added to the melt, the reaction proceeded at a rapid rate and the yields of urea were satisfactory.   The synthesis of Hentschel is thus catalyzed by copper, and it is probable that Hentschel in 1884, at a time when large glass or porcelain vessels were not easily obtainable, carried out the procedure in a copper bulb.

[10] In an experiment, carried out for other purposes and not included in the tables, in which excessive doses of urea (100 mg. per day) were given to rats, the creatine had *a very small isotope content (0.014 atom per cent excess)*; *i.e.*, a minute amount of the nitrogen given as urea was present in the creatine.   However, the feeding of such large quantities makes it difficult to interpret the biological findings.   Part of the urea might have been degraded in the intestinal tract to ammonia, and the ammonia might

Choline—This compound has been considered by Riesser (34) to be a creatine precursor. Its metabolism is known, from recent reports (35–37), to be intimately connected with the metabolism of methionine, which yields its methyl group for creatine formation (12, 13). While choline is stated by Borsook and Dubnoff (12) not to yield its methyl group directly to guanidoacetic acid, it might do so, at least indirectly, via methionine.

This paper is not concerned with the transfer of methyl groups; our results with isotopic nitrogen cannot reveal their fate. According to the schemes proposed by Riesser, however, the nitrogen of choline is also utilized for creatine formation. We have prepared choline containing the nitrogen isotope. Isotopic ethanolamine was synthesized by condensing isotopic potassium phthalimide with ethylene bromide (38) and hydrolysis of the product with alkali (39). The base was converted into choline by methylation with dimethyl sulfate.

The creatine isolated after administration of this choline preparation had an isotope content only slightly above the error of the analytical procedure. The choline nitrogen is thus not employed directly for biological creatine synthesis.

Sarcosine—By treatment with alkali creatine and creatinine are degraded into ammonia, carbon dioxide, and sarcosine. The function of the latter as a creatine precursor has frequently been discussed. Like almost all other compounds, but unlike guanidoacetic acid, its administration does not increase creatine output (40).

Sarcosine has recently been shown by Abbott and Lewis (41) to be able to replace glycine as a precursor of hippuric acid, indicating its rapid conversion to glycine. A similar result has been obtained with isotopes (42). The feeding of isotopic sarcosine to rats is followed by almost the same distribution of isotope among the various amino acids of the proteins as is found when glycine is given. As the glycine of the proteins of the animals has a very high isotope content, demethylation of the sarcosine to glycine must have proceeded at a very rapid rate.

The creatine isolated after sarcosine feeding had an isotope

---

have entered the creatine via the amidine group of arginine. Even if the result of this experiment were accepted as showing the utilization of urea for creatine formation (which we are not inclined to do), urea would still have to be considered as the most ineffective of the established precursors.

content slightly lower than that observed after the feeding of an equivalent amount of glycine. Sarcosine is therefore less effective than it should be if it represented one of the intermediate stages in creatine synthesis between glycine and creatine. The formation

TABLE I

N^{15} Content in Muscle Creatine after Administration of Isotopic Compounds

Compound administered	Isotope content	Total nitrogen administered per day	Duration of experiment	Isotope content in		
				Total creatine or creatinine	Amidine group	Sarcosine group
	atom per cent excess	mg.	days	atom per cent excess	atom per cent excess	atom per cent excess
dl-Tyrosine..	2.00	14.4	10	0.001	0.008	
l(−)-Leucine	6.50	23	3	0.009	0.011	
dl-Glutamic acid . . .	4.5	25	3	0.008		
Ammonium citrate. ...	2 00	26.4	9	0.009		
" " + 300 mg. benzoic acid . .	1.21	72	6	0.025		
Ammonium citrate on protein-low diet* .	1 21	180	5	0.036	0.044	0.014
Glycine .. .	4 5	23	3	0.030	0.006	0.070
"	4.5	25	6	0.078	0.016	0.205†
"*	4.5	25	6	0.225	0.049	0.515
Sarcosine	4.5	23	3	0.021		
"	4.5	23	6	0.055	0.016	0.133†
Arginine....	8.3	23	3	0.108	0.130	0.003
Guanidoacetic acid.. ..	1.5	4.2	6	0.128		
" " *.	1.5	4.5	6	0.528		
Hydantoic acid*......	2.25	6	6	0.003		
Methylhydantoic acid*....	2.25	5.2	6	0.003		
Urea.	15.2	23	3	0.003		
Choline*.	2.00	19	3	0.009		

* Immature rats.
† The value was not determined but calculated.

of isotopic creatine from isotopic sarcosine is thus only additional evidence of the rapid biological demethylation of sarcosine.

Relative Efficiency of Nitrogenous Compounds As Creatine Precursors

The efficiency of an isotopic compound as a creatine precursor is indicated in our experiments by the N^{15} content in the isolated

creatine or in its two nitrogenous groupings.   The isotope content in the creatine samples isolated (Table I) is dependent upon a variety of factors, which include (1) the rate of creatine formation in the animal, (2) the duration of the experiment, and (3) the isotope concentration (in atom per cent N^{15} excess) of the compound administered.

TABLE II

Fraction of Newly Formed Creatine Nitrogen Derived from Respective Test Substance

Material administered	Fraction of N derived from test substance		
	Of total creatine	Of amidine group	Of sarcosine group
	per cent	*per cent*	*per cent*
dl-Tyrosine . .	0		
l-Leucine	2 3	2 8	
dl-Glutamic acid	3 3		
Ammonia*	2.5		
Glycine* , ...	11.1	2.2	25.9
" , . ..	14.4	3.0	
Sarcosine ...	7.7		
"	10.2	3.0	
Arginine.	22.0	26 4	0.6
Hydantoic acid	0		
Methylhydantoic acid	0		
Urea	0		
Choline	0		

Guanidoacetic acid is not included in the table, as its administration is known to result in extra creatine formation.   The efficiency of this compound calculated by employing the same factor would be 66.7 per cent.

* Only those experiments were considered in which adult rats were employed.

The conditions varied widely from experiment to experiment. The periods varied from 3 to 10 days, and the isotope concentrations in the administered compounds varied from 1.2 to 15.2 atom per cent.   The rate of creatine formation in adult animals was probably constant within narrow limits except when guanidoacetic acid was given.

In order to compare the results of the various experiments the values obtained (Table I) have to be reduced to the same basis. In Table II are calculated for each experiment the fractions of the

newly formed creatine (or its two groupings) which had been derived from the nitrogen of the test substance. The assumption was made on the basis of our previous determination (11) that in each experiment 2 per cent of the creatine of the adult animals was newly formed per day.

The values in Table II were calculated by the following formula from the values given in Table I

$$\frac{b \times 100}{a \times t \times 0.02}$$

where a = the isotope concentration (in atom per cent) of the material administered, b = the isotope concentration (in atom per cent) in creatine (or its groupings), and t = days of experiment.

Table II indicates the efficiency of each compound as a creatine former. 26 per cent of the nitrogen in the sarcosine group of creatine was derived from the isotopic glycine and about the same fraction of the amidine nitrogen was derived from the amidine group of the isotopic arginine. None of the values could have reached 100 per cent, even if the nitrogen of glycine and arginine were the only sources for creatine synthesis. The isotopic compounds added to the diet had rapidly merged with the same non-isotopic compounds derived from the dietary casein and the proteins of the animals. The nitrogen that was utilized must thus have been a mixture of isotopic and non-isotopic material. If glycine and arginine had been the only sources, the values found would indicate that the isotopic glycine had been mixed in the metabolism with 4 times its weight of ordinary glycine, and the isotopic arginine with about 4 times its weight of ordinary arginine before either had reached the site of creatine formation. On the basis of recent experiments which demonstrated the rapidity with which dietary and tissue constituents interact, such an assumption seems reasonable. However, as we do not know exactly the extent of this mixing process, we cannot state definitely that glycine and arginine are the *only* sources for creatine nitrogen. As the other compounds investigated showed little or no effect, the nitrogen of glycine and that of the amidine group of arginine obviously represent the main sources of the nitrogen of creatine.

Biological Synthesis of Creatine

The present results, in conjunction with those on the methyl transfer (12, 13), seem to establish the immediate biological origin of every part of the creatine molecule (V).

From proteins   From arginine   From methionine

$$
\begin{array}{ccccc}
& & & & NH_2 \\
& & NH_2 & & | \\
\downarrow & \downarrow & | & \downarrow & C{=}NH \\
NH_2 & & C{=}NH & & | \\
| & + \ H_2N{-}C{=}NH \ \rightarrow & | & + {-}CH_3 & N{-}CH_3 \\
CH_2COOH & | & NH & \xrightarrow{\qquad} & | \\
& & | & & CH_2COOH \\
& & CH_2COOH & &
\end{array}
$$

(V)
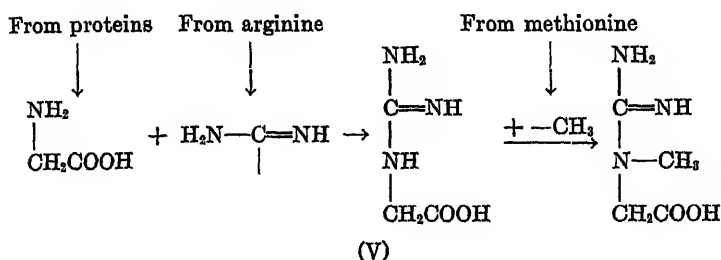
Not only the precursors but also the sequence of the condensation reaction seems to be established. Methylation cannot have occurred before the condensation of glycine with the amidine group, as guanidoacetic acid is an intermediate, while sarcosine is not.

Creatine As a Measure of Endogenous Metabolism

Folin (43), in his classical study of creatine metabolism, put forward evidence according to which creatinine excretion is independent not only of protein intake but also of creatine. He contended that creatinine despite its chemical relation to creatine is metabolically independent of it. He assumed that creatinine, like urea, is a metabolic waste product but suggested that both originate from different types of protein catabolism, one of which (resulting in creatinine, etc.) is constant, while the other (resulting in urea and ammonia) is dependent upon the amount of protein intake. In accordance with the concept, prevalent at that time, that the food material, including amino acids, is directly degraded for energy requirements, whereas the structural body components are mainly stationary, he attributed urea formation to the catabolism of food constituents (variable or exogenous metabolism) and creatinine to that of the tissue proteins (constant or endogenous metabolism). Creatinine formation, according to Folin, probably represents "an essential part of the activity which distinguishes living cells from dead ones."

The interpretation of some of the experimental results on which Folin's hypothesis is based has since been found to be incorrect. Creatinine excretion is not independent of creatine intake, as the feeding of creatine is followed by a delayed extra excretion of creatinine (44).

Recently (15) the normal creatinine of the urine has been demonstrated to be derived directly from body creatine and its nitrogen must be regarded as representative of that of the tissue creatine. Both compounds must thus have identical sources.

The present study has shown that a considerable fraction of the creatine nitrogen (and therefore also of the creatinine nitrogen) is provided by normal food constituents; i.e., glycine and arginine. As much as 25 to 30 per cent of the nitrogen in the newly formed creatine had been derived from the isotopic compounds added to the casein-containing stock diet, and an additional part of the creatine nitrogen must have been derived from the same, non-isotopic, amino acid in the dietary proteins. The remainder of the creatine nitrogen must have been supplied by the arginine and glycine of the tissues. Creatine and creatinine are thus constantly formed from food as well as tissue material. The present findings on the sources of creatine (and therefore also of creatinine) provide additional evidence against a concept of two separate types of catabolism.[11]

<center>EXPERIMENTAL</center>

Isotopic guanidoacetic acid was prepared according to Strecker (14). To 0.75 gm. of glycine (4.5 atom per cent N^{15} excess) in 5 cc. of water were added 0.84 gm. of non-isotopic cyanamide and 3

---

[11] In a recent paper by Burroughs, Burroughs, and Mitchell (45) the theory of Folin, including the concept of creatinine as a measure of endogenous metabolism, is upheld and the findings of this laboratory on the rapid chemical interactions of dietary amino acids and tissue proteins are given a new interpretation: The chemical activity of the body proteins is restricted to the "dispensable reserve proteins" and does not involve the "fixed" proteins. According to this concept one would thus have to distinguish between the catabolism of food and dispensable reserve proteins on the one hand and that of specific cell constituents on the other. Acceptance of this interpretation would compel, as a corollary of the present findings, the view that creatine and creatinine are formed from food and "dispensable reserve proteins." This would lead to the hardly acceptable conclusion that they are derived from "exogenous" metabolism only.

drops of concentrated ammonia.   The mixture was kept at room temperature for 3 days.   The crystalline precipitate was filtered off and recrystallized from a large volume of water.   The yield was 0.75 gm.

N (Kjeldahl) calculated, 35.9; found, 36.0
N^{15} calculated, 1.50 atom per cent excess; found, 1.50

Isotopic hydantoic acid was prepared according to West (16). 0.525 gm. of glycine (4.5 atom per cent N^{15} excess) and 0.61 gm. of non-isotopic potassium cyanate were dissolved in 20 cc. of water and the mixture heated on the steam bath for 1 hour.   The solution was filtered, cooled, and made acid to Congo red by addition of concentrated hydrochloric acid.   The crystalline precipitate was recrystallized from a small volume of water. The yield was 0.50 gm.

N (Kjeldahl) calculated, 23.7; found, 23.8
N^{15} calculated, 2.25 atom per cent excess; found, 2.26

Isotopic N-methylhydantoic acid was prepared according to Salkowski (17). 0.446 gm. of sarcosine (4.5 atom per cent N^{15} excess) and 0.41 gm. of normal potassium cyanate in 7 cc. of water were heated together on the steam bath for 5 minutes.   The mixture was cooled, and acidified by addition of hydrochloric acid.   The crystalline precipitate was filtered off and recrystallized from a small volume of water.   The yield was 0.33 gm.

N (Kjeldahl) calculated, 21.2; found, 21.2
N^{15} calculated, 2.25 atom per cent excess; found, 2.25

Preparation of Isotopic Arginine

α-p-Toluenesulfonylornithine—*α-p*-Toluenesulfonylarginine was prepared from *l*(+)-arginine monohydrochloride (26).   The compound had a rotation of $[\alpha]_D^{22} = -15.0°$ (3.3 per cent in 15 per cent hydrochloric acid).   43 gm. were dissolved in a solution containing 350 gm. of crystalline barium hydroxide in 2100 cc. of water and boiled under a reflux for 21 hours, when no more ammonia was liberated.   The barium was removed by addition of exactly the necessary amount of sulfuric acid, and filtrate and washings were brought to dryness *in vacuo*.   The dry residue was extracted several times with 70 per cent acetone and the combined

extracts concentrated to a small volume. The crystals obtained by cooling were recrystallized from 85 per cent ethanol. The yield was 26 gm. (79 per cent of the calculated amount) of toluene-sulfonylornithine, m. p. 207°.

N (Kjeldahl) calculated, 9.8; found, 9.7
S calculated, 11.2; found, 11.0
$[\alpha]_D^{24} = -3.15°$ (6.5% in 15% hydrochloric acid)

The product so obtained had been partially racemized. It has a lower rotation than a sample of toluenesulfonylornithine which was obtained by the action of arginase upon toluenesulfonyl-arginine. This latter substance also melted at 207°, but had a rotation of $[\alpha]_D^{25} = -5.5°$ (2 per cent in 15 per cent hydrochloric acid).[12]

Isotopic O-Methylisourea—Isotopic cyanamide was prepared from non-isotopic cyanogen bromide and isotopic ammonia (34.0 atom per cent N^{15} excess) as described before (11). The isotopic cyanamide was converted into O-methylisourea hydrochloride according to Stieglitz (46), and the free base was obtained from the hydrochloride according to Kapfhammer and Mueller (47).

Isotopic Toluenesulfonyl-l(+)-Arginine—The solution of 2.8 gm. of O-methylisourea in 25 cc. of methanol was added to 7.5 gm. of toluenesulfonylornithine, dissolved in a mixture of 75 cc. of water and 220 cc. of methanol. The procedure was thus similar to that of Kapfhammer (47) for the preparation of other guanido acids. The mixture was treated with 3 cc. of concentrated non-isotopic ammonia and kept in the ice box for a week. The crystalline precipitate weighed 4.7 gm. (Fraction A). On concentration of the filtrate a second crop, 2.5 gm. (Fraction B), was obtained. The total yield of crude toluenesulfonylarginine was 7.2 gm., or 82 per cent of the calculated amount.

Fraction A consisted mainly of the *dl* component, with a rotation of $[\alpha]_D^{24} = -1.8°$ (4 per cent in 15 per cent hydrochloric acid). Repeated crystallizations by dissolving in an equivalent amount

[12] As we had obtained only a small amount (60 mg.) of this sample, the value of the rotation is not very precise. The enzymatic degradation of toluensulfonylarginine is not practical, as it requires very large amounts of enzyme. We are indebted to Dr. M. Richards of this department for a sample of purified arginase.

of hydrochloric acid and neutralizing with sodium carbonate did not accomplish complete removal of the l isomer. After three crystallizations the rotation was $[\alpha]_D^{24} = -0.7°$ (4 per cent in 15 per cent hydrochloric acid). The compound started to decompose slowly at 265°. It is very slightly soluble in water.

Calculated, N 17.1, S 9.8; found, N 16.9, S 9.9

Fraction B melted at 256°. After one recrystallization from water it had a rotation of $[\alpha]_D^{23} = -15.1°$ (2 per cent in 15 per cent hydrochloric acid); *i.e.*, the same rotation as that of toluenesulfonyl-$l(+)$-arginine prepared by direct toluenesulfonylation of l-arginine. Thus, the more water-soluble reaction product consisted of toluenesulfonyl-$l(+)$-arginine, whereas the less soluble fraction represented mainly the dl component.

Isotopic $l(+)$-Arginine Monohydrochloride—For the removal of the toluenesulfonyl group the l and the dl fractions were hydrolyzed separately by heating with concentrated hydrochloric acid in sealed tubes on the steam bath for 36 hours. The reaction mixture was brought to dryness *in vacuo*, the residue taken up in water, and a slight excess of flavianic acid was added. From 3.23 gm. of dl-toluenesulfonylarginine there were obtained 4.68 gm. of dl-arginine monoflavianate, corresponding to a yield of 96 per cent, and from 2.44 gm. of l-toluenesulfonylarginine 3.3 gm. of l-arginine monoflavianate, corresponding to a yield of 90 per cent.

Flavianic acid was removed from the l salt by suspension in concentrated hydrochloric acid and filtering off the flavianic acid. The filtrate was diluted with water, treated with norit, and brought to dryness *in vacuo*. The residue was taken up in 95 per cent ethanol and pyridine was added. The yield after recrystallization from water-ethanol was 1.20 gm. of $l(+)$-arginine monohydrochloride or 77 per cent of the amount calculated from the weight of toluenesulfonylarginine taken.

Calculated, C 34.1, H 7.2, N 26.7; found, C 34.0, H 7.0, N 26.9
$[\alpha]_D^{23} = +24.9°$ (1.2% in 15% hydrochloric acid) calculated for free arginine

In the course of the arginine synthesis the isotopic ammonia (34.0 atom per cent N^{15} excess) originally employed for the synthesis of O-methylisourea had been diluted with 3 atoms of

non-isotopic nitrogen (a) by 1 atom from the non-isotopic cyanogen bromide and (b) by 2 atoms from the non-isotopic toluenesulfonyl-ornithine. The isotope concentration in the arginine should therefore be 34.0/4 = 8.5 per cent. The concentration found was 8.3 atom per cent N^{15} excess.

Isotopic Ethanolamine—Bromoethylphthalimide was prepared as described in "Organic syntheses" (38) from 37 gm. of isotopic potassium phthalimide (2.0 atom per cent N^{15} excess) and 90 gm. of ethylene dibromide. The yield of bromoethylphthalimide was 33.8 gm. or 67 per cent of the calculated amount. This was subjected to alkaline hydrolysis with 30 per cent potassium hydroxide solution according to Putokhin (39). The ethanol-amine was distilled from the reaction mixture into an excess of dilute hydrochloric acid. Ethanolamine hydrochloride was isolated from its alcoholic solution by precipitation with ether. The yield was 9.23 gm., or 71 per cent.

> Calculated. N (Kjeldahl) 14.4, (amino) 14.4
> Found.   "   "   14.6,   "   14.3
> N^{15} calculated, 2.0 atom per cent excess; found, 2.0

Isotopic Choline—To a cooled solution of 3.9 gm. of ethanol-amine hydrochloride (2.0 atom per cent N^{15} excess) in 170 cc. of 5 per cent potassium hydroxide solution, 25.5 gm. of dimethyl sulfate were gradually added with stirring. The stirring was continued for 1 hour and the filtered solution made acid to Congo red with hydrochloric acid. On the addition of 15 gm. of gold chloride, 14.2 gm. of choline gold chloride were obtained, or 80 per cent of the calculated amount. The substance melted at 268° with decomposition.

> N (Kjeldahl) calculated, 3.16; found, 3.17
> N^{15} calculated, 2.0 atom per cent excess; found, 2.0

Isotopic Urea—The compound was prepared according to Hentschel (33). 2.72 gm. of diphenyl carbonate and 50 mg. of copper dust were placed in a steam-jacketed shaking flask. The flask, while being heated to 100°, was evacuated and connected with a vessel in which ammonia was generated from 1.70 gm. of isotopic ammonium chloride (15.3 atom per cent N^{15} excess). The uptake of ammonia was complete after 30 minutes. The

reaction mixture was taken up in water and extracted twice with chloroform. The aqueous solution was treated with norit, filtered, and brought to dryness *in vacuo*. The residue was taken up in a small volume of ethanol and precipitated with benzene. The crystalline precipitate of urea was further purified by extraction from a thimble with acetone in a Soxhlet apparatus. The urea crystallized from acetone weighed 0.44 gm., or 58 per cent of the calculated amount. The compound melted at 132–133°.

N (Kjeldahl) calculated, 47.0; found, 47.0
N^{15} calculated, 15.3 atom per cent excess; found, 15.2

Feeding Experiments

The experimental conditions employed in the feeding of the following substances have been described before: ammonia (21, 22), glycine (27), sarcosine (42), *dl*-tyrosine (48), *l*-leucine (49). In all these experiments muscle creatine and urinary creatinine were isolated by the methods previously reported (15). The analytical values are given in Table I. All animals received the same stock diet (15).

Glycine Feeding to Immature Rats—Two growing rats, having a combined weight of 185 gm., received 25 mg. of glycine nitrogen (4.5 atom per cent N^{15} excess) each day for 6 days, when they were killed. At the end of the feeding period the animals had a combined weight of 246 gm. Muscle creatine was isolated and degraded into ammonia and sarcosine by baryta.

Guanidoacetic Acid Feeding I—Two adult rats, having a combined weight of 568 gm., were given 11.7 mg. of guanidoacetic acid (1.5 atom per cent N^{15} excess) per day per rat for 6 days. The animals were then killed and the muscle creatine isolated.

Guanidoacetic Acid Feeding II—Two growing rats, having a combined weight of 179 gm., received 25 mg. of guanidoacetic acid (1.5 atom per cent N^{15} excess) per rat per day for 6 days. The animals gained a total of 55 gm. After the animals had been killed, muscle creatine was isolated. The isotope concentration was also determined in the total urinary nitrogen excreted during the feeding period as well as in the urinary urea.

Total urinary nitrogen, 0.116 atom per cent N^{15} excess
Urea, 0.004 atom per cent N^{15} excess

The fact that a considerable amount of the administered isotope was excreted, but was not present in the urea, suggests that part of the guanidoacetic acid was excreted unchanged. This is also indicated by the strongly positive Sakaguchi reaction in the urines. These results are in agreement with those of Jaffe (50) who showed that an excess of guanidoacetic acid is not metabolized but excreted as such.

Hydantoic Acid Feeding—One growing rat, weighing 92 gm., received 25 mg. of hydantoic acid (2.25 atom per cent N^{15} excess) prepared from isotopic glycine (4.5 atom per cent N^{15} excess) and non-isotopic potassium cyanate, per day for 6 days, when it was killed. The animal had gained 24 gm. Muscle creatine was isolated and found to contain no excess of isotope.

Methylhydantoic Acid Feeding—One growing rat, weighing 102 gm., received 25 mg. of methylhydantoic acid (2.25 atom per cent N^{15} excess) prepared from isotopic sarcosine and non-isotopic potassium cyanate, per day for 6 days, when it was killed. The animal had gained 20 gm. The isolated muscle creatine did not contain excess of isotope.

Choline Feeding—Three immature rats, weighing 199 gm., received 19 mg. of choline nitrogen (2.0 atom per cent N^{15} excess) per day per rat for 3 days. The choline was obtained from choline aurichloride by removing the gold with hydrogen sulfide. At the end of the feeding period the animals weighed 208 gm. They were killed and the muscle creatine isolated.

Urea Feeding—Two adult rats, having a combined weight of 557 gm., received 23 mg. of urea nitrogen (15.2 atom per cent N^{15} excess) per day per rat for 3 days. Muscle creatine was isolated and found to contain no excess of isotope.

Feeding of l(+)-Arginine—Two adult rats, having a combined weight of 646 gm., received 23 mg. of arginine nitrogen (8.3 atom per cent N^{15} excess) per rat per day for 3 days. The animals were then killed and creatine was isolated from the muscles. The creatine sample was degraded with barium hydroxide and isotope analysis was made of the ammonia and the sarcosine. The values are given in Table I.

<div align="center">SUMMARY</div>

1. The formation of creatine in normal rats was investigated by adding to their ordinary stock diet twelve different compounds

containing the nitrogen isotope. The substances tested were (1) ammonia, (2) *dl*-tyrosine, (3) *l*(−)-leucine, (4) *dl*-glutamic acid, (5) glycine, (6) *l*(+)-arginine, (7) sarcosine, (8) guanido-acetic acid, (9) hydantoic acid, (10) methylhydantoic acid, (11) choline, (12) urea. The preparation of Compounds 6 and 8 to 12 is described. At the end of the feeding period creatine samples were isolated from tissues of the animals and their isotopic content was determined.

2. In order to locate the position of the isotopic nitrogen among the nitrogen atoms of the isolated creatine, some of the samples were degraded with barium hydroxide, whereby the nitrogen of the amidine group was recovered as ammonia, and the remaining nitrogen as sarcosine.

3. Urea, hydantoic acid, methylhydantoic acid, and choline are not creatine precursors. Creatine isolated after feeding these compounds did not contain the isotope marker.

4. Arginine and glycine are the only natural amino acids investigated so far which are potent precursors of creatine; their feeding results in the formation of creatine with a high isotope content. Each of the two compounds supplies nitrogen to different parts of the creatine molecule; glycine is utilized for the sarcosine moiety; the amidine group of arginine supplies the amidine group of creatine.

5. After the feeding of isotopic ammonia and of the isotopic amino acids tyrosine, leucine, and glutamic acid, the creatine has only a very low concentration of isotope. The ammonia, when given in very large amounts, yields creatine with an isotope concentration high enough for degradation studies. Most of the isotope was found to be present in the amidine part of the creatine; *i.e.*, the effect of these compounds on creatine formation is qualitatively the same as that of feeding arginine with an isotopic amidine group. These substances are only indirect creatine precursors: they yield nitrogen to the amidine group of the arginine of proteins which in turn is shifted to glycine to form guanidoacetic acid. A small amount of isotopic glycine, formed with the nitrogen from these compounds, is also employed for creatine synthesis.

6. Interpretation of the quantitative data reveals that in the present experiments a minimum of 26 per cent of the amidine nitrogen of the newly formed creatine was derived from that of the

added arginine, and 26 per cent of sarcosine nitrogen of the creatine was derived from the added glycine.

7. The hypothesis that guanidoacetic acid is an intermediate in creatine formation is corroborated by the finding that the feeding of isotopic guanidoacetic acid results in creatine with a very high isotope content. Guanidoacetic acid is the most potent creatine precursor investigated so far.

8. Sarcosine, while almost as effective as a precursor of creatine as is glycine, cannot be considered as an intermediate. Its effectiveness is due to its rapid demethylation to glycine.

9. The present findings, in conjunction with the recent observation of other laboratories, according to which the methyl group of creatine is supplied by methionine, establish the immediate biological origin of all parts of the creatine molecule, as well as the sequence of their condensations. Glycine reacts with the amidine group of arginine to form guanidoacetic acid, and this is methylated to form creatine by shift of the methyl group from methionine.

10. The finding that the newly formed creatine (and creatinine) molecules acquire their parts from food as well as from tissue components is taken as further evidence against the concept of two independent (exogenous and endogenous) types of catabolism.

Addendum

The present findings on the sources of creatine cannot be reconciled with the experimental results or with the theory of Beard et al. (32, 51, 52). According to Beard and Pizzolato (32) the injection of amino acids (and some other nitrogenous compounds) into rats results in a large increase of muscle creatine. These findings in conjunction with some others were interpreted as showing that the natural amino acids are converted into glycine and urea, both of which condense to form guanidoacetic acid, which in turn is methylated by another molecule of glycine to form creatine. The experiments could not be reproduced by other investigators (53), and are also in conflict with the general belief that the rate of creatine formation is almost independent of the amount of dietary amino acids.

Beard and Pizzolato (32) did not compute the actual amounts of creatine formed by their rats from injected amino acids. We

have calculated from their values how much of the nitrogen of the administered amino acid must have been utilized for creatine formation. This was based on the following data. The authors state the amount of amino acids in mg. administered and the resulting increase of muscle creatine in per cent of preformed creatine. The weights of the animals were given as 100 to 250 gm., and they assumed in their own calculations that the average

TABLE III

Utilization of Amino Acids for Creatine Formation According to Beard and Pizzolato (32)

Compound injected (1)	N injected (2)	Increase of muscle creatine observed (3)	Increase of creatine N calculated (4)	Utilization of injected N for creatine formation (5)	Values calculated for 1 nitrogen atom in creatine (6)
	mg.	*per cent*	*mg.*	*per cent*	*per cent*
α-Aminobutyric acid, 200	27	38	39	143	48
α-Aminocaproic acid, 200..	21	45	46	216	72
α-Aminovaleric acid, 200..	24	52	54	224	75
Aspartic acid, 200 .	21	22	23	108	36
Cystine, 100 .	12	19	20	166	56
Glutamic acid, 200	19	18	19	100	33
Hydantoin, 100	28	82	83	296	99
Glutathione, 66.	9	40	41	455	151
Leucine, 200..	21	15	15	71	24
Lysine, 200	38	17	18	46	15
Ornithine, 100.	21	63	65	306	102
Phenylalanine, 200	17	15	16	94	31
Valine, 100.... ..	12	22	23	192	64
Glycine, 200.	37	32	33	89	30
Glycylglycine, 200.	42	81	83	193	64
Guanidoacetic acid, 200.. .	72	28	29	40	13
Arginine, 200. . .	64	37	38	59	20
Urea, 300	140	29	30	21	7

weight of their rats was 200 gm. This value was also taken for our calculations. They consider such rats to contain 80 gm. of muscle tissue, and the rat muscle was found by them to contain 0.40 per cent creatine. The average rat employed for their studies thus contained a total of 320 mg. of muscle creatine, or 102 mg. of creatine nitrogen. If, as was found by these authors, 200 mg. of aminobutyric acid increase the content of muscle creatine by 38

per cent, this means that 27 mg. of injected amino nitrogen have produced 39 mg. of creatine nitrogen, or 143 per cent. The results of some of the calculations are given in Column 5 of Table III. We have considered in each series only those positive experiments in which the smallest amount of compound was injected.

According to these values most compounds have yielded much more than 100 per cent of their nitrogen for creatine formation. It is interesting to note that urea, glycine, and guanidoacetic acid are according to these calculations relatively ineffective. If only 1 nitrogen atom of the creatine be assumed to have had its origin in the injected compound, the amount of nitrogen utilized for creatine formation would be only one-third of these values. The resulting figures (Column 6) are still so high as to suggest that creatine formation (and not urea formation, as is generally believed) is one of the main end-products of the metabolism of most amino acids.

Beard and Pizzolato (54) still hold to their contention that creatine is *not* converted into creatinine, but that the reverse process occurs; *i.e.*, formation of creatine from creatinine. They base their claim on experiments of Beard and Jacob (55), who showed that injection of as little as 10 mg. of creatinine into rats results in an increase of muscle creatine by 19 per cent. This, according to the above calculation, would correspond to 60.8 mg. of creatine, or 520 per cent of the theory.

BIBLIOGRAPHY

1. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 633 (1940).
2. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 785 (1940).
3. Rose, W. C., in Luck, J. M., Annual review of biochemistry, Stanford University, **4**, 258 (1935).
4. Brand, E., Harris, M. M., and Ringer, A. J., *Am. J. Physiol.*, **90**, 296 (1929).
5. Fisher, R. B., and Wilhelmi, A. E., *Biochem. J.*, **31**, 1136 (1937).
6. Hunter, A., Creatine and creatinine, Monographs on biochemistry, London and New York (1928).
7. Thomas, K., in Luck, J. M., Annual review of biochemistry, Stanford University, **7**, 211 (1938).
8. Borsook, H., and Dubnoff, J. W., *Science*, **91**, 551 (1940).
9. Weber, C. J., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **114**, p. cvii (1936).
10. Bodansky, M., *J. Biol. Chem.*, **115**, 641 (1936).

11. Bloch, K., Schoenheimer, R., and Rittenberg, D., *J. Biol Chem.*, **138**, 155 (1941).
12. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940).
13. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).
14. Strecker, A., *J. prakt. Chem.*, 530 (1861).
15. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **131**, 111 (1939).
16. West, C. J., *J. Biol. Chem.*, **34**, 187 (1918).
17. Salkowski, E., *Ber. chem. Ges.*, **7**, 116 (1874)
18. Lewis, H. B., *J. Biol. Chem.*, **13**, 347 (1912–13).
19. Baumann, L., and Marker, J., *J. Biol. Chem.*, **22**, 49 (1915).
20. Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, **135**, 153 (1940).
21. Rittenberg, D., Schoenheimer, R., and Keston, A. S., *J. Biol. Chem.*, **128**, 603 (1939).
22. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
23. Czernecki, W., *Z. physiol. Chem.*, **44**, 294 (1905).
24. Davenport, H. W., Fisher, R. B., and Wilhelmi, A. E., *Biochem. J.*, **32**, 262 (1938).
25. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **172**, 277 (1927).
26. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939).
27. Ratner, S., Rittenberg, D., Keston, A. S., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 665 (1940).
28. Abderhalden, E., and Buadze, S., *Z. ges. exp. Med.*, **65**, 1 (1929).
29. Schoenheimer, R., Rittenberg, D., and Keston, A. S., *J. Biol. Chem.*, **127**, 385 (1939).
30. Cox, G. J., and Rose, W. C., *J. Biol. Chem.*, **68**, 781 (1926).
31. Zwarenstein, H., *Biochem. J.*, **20**, 742 (1926).
32. Beard, H. H., and Pizzolato, P., *J. Biochem.*, Japan, **28**, 421 (1938).
33. Hentschel, W., *Ber. chem. Ges.*, **17**, 1284 (1884).
34. Riesser, O., *Z. physiol. Chem.*, **86**, 415 (1913).
35. Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, **121**, 479 (1937).
36. du Vigneaud, V., Dyer, H. M., and Kies, M. W., *J. Biol. Chem.*, **130**, 325 (1939). Rose, W. C., and Rice, E. E., *J. Biol. Chem.*, **130**, 305 (1939).
37. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
38. Salzberg, P. L., and Supniewski, J. V., in Gilman, H., *et al.*, Organic syntheses, New York, coll. **1**, 114 (1932).
39. Putokhin, N., *Tr. Inst. Pure Chem. Reagents*, **6**, 10 (1927); *Chem. Abst.*, **23**, 2938 (1927).
40. Riesser, O., *Z. physiol. Chem.*, **86**, 415 (1913).
41. Abbott, L. D., Jr., and Lewis, H. B., *J. Biol. Chem.*, **131**, 479 (1939).
42. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 99 (1940).
43. Folin, O., *Am. J. Physiol.*, **13**, 117 (1905).
44. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, **56**, 229 (1923).

45. Burroughs, W. W., Burroughs, H. S., and Mitchell, H. H., *J. Nutrition*, **19**, 271 (1940).
46. Stieglitz, J., *Ber. chem. Ges.*, **33**, 517 (1900).
47. Kapfhammer, J., and Mueller, H., *Z. physiol. Chem.*, **225**, 1 (1932).
48. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **127**, 333 (1939).
49. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).
50. Jaffe, M., *Z. physiol. Chem.*, **48**, 430 (1906).
51. Beard, H. H., and Boggess, T. S., *J. Biol. Chem.*, **114**, 771 (1936)
52. Beard, H. H., and Espenan, J. K., *Enzymologia*, **8**, 241 (1940).
53. Fisher, R. B., and Wilhelmi, A. E., *J. Biol. Chem*, **132**, 135 (1940).
54. Beard, H. H., and Pizzolato, P., *Endocrinology*, **27**, 908 (1940).
55. Beard, H. H., and Jacob, E. J., *Arch. Neurol. and Psychiat.*, **42**, 67 (1939).

THE EFFECT OF CERTAIN INHIBITORS AND ACTIVATORS ON SPERM METABOLISM*

By HENRY A. LARDY and PAUL H. PHILLIPS

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

The studies reported herein are a continuation of our work on semen storage for artificial insemination purposes (1, 2), and were undertaken to elucidate further the mechanism by which spermatozoa obtain energy for motility. By means of inhibitors we hoped to gain information on various systems involved in obtaining this energy.

Methods

The semen used in these studies was collected from healthy dairy bulls by means of an artificial vagina and was kept in a refrigerator until used. All experiments were begun within 2 hours after the semen was collected.

Observations were made on the effect of inhibitors on bull sperm in the egg yolk-buffer pabulum previously described (2, 3). Semen and yolk-buffer at pH 6.8 were mixed at the ratio of 1:3 and stored at 10°. Motility and lactic acid production were studied in a modified Ringer-phosphate medium of the following composition: 0.9 per cent NaCl 100 parts, 1.15 per cent KCl 4 parts, 2.11 per cent KH_2PO_4 1 part, 0.25 M Na_2HPO_4 20 parts. Enough 1 N HCl was added to bring the mixture to pH 6.8. When this medium was used, the spermatozoa were removed from the seminal fluid by centrifuging and washed by suspending in 0.9 per cent NaCl. After centrifuging again the spermatozoa

were suspended in the Ringer-phosphate solution at the dilution desired. Glucose was added to give a final concentration of 0.04 M.

All inhibitor and activator solutions were adjusted to pH 6.8 before use.

Motility observations were made with a microscope (magnification 440 ×) equipped with a warm stage at 37.2–37.8°. The degree of motility was recorded as 1+ to 5+ depending on the percentage of the spermatozoa that was actively motile. 5+ represented excellent motility, 1+ less than 10 per cent motile, and F. M. indicated few progressively motile spermatozoa.

Lactic acid, in protein- and carbohydrate-free filtrates, was determined by an adaptation of the colorimetric method of Mendel and Goldscheider (4) similar to that of Barker (5). Proteins were precipitated with metaphosphoric acid and interfering substances removed by the usual copper-lime treatment. Although high levels of iodoacetic acid may interfere with the determination of lactic acid (6), the concentrations used in our work did not affect the accuracy of the method.[1]

Results

Before the action of inhibitors was studied, a preliminary survey was made of some of the enzyme components present in semen and spermatozoa. It was found that succinic dehydrogenase, an alkaline phosphatase, and riboflavin were present. With a hand spectroscope it was impossible to detect the absorption bands of reduced cytochromes in homogenized sperm treated with hydrosulfite.

By means of the Thunberg technique an active succinic dehydrogenase was found to be present in spermatozoa. Results with various substrates are shown in Table I. These are typical of several such experiments. Of special interest was the observation that the reduction time with glucose or lactate was longer than the reduction time with no added substrate.

An alkaline phosphatase whose activity was from 50 to 100 times as great as that present in blood plasma was found in semen. The enzyme was found in both the sperm and the seminal fluid with by far the greater concentration per unit volume in the sperm.

[1] Lardy, H. A., and Phillips, P. H., unpublished data.

Highly purified, stable preparations of this enzyme have been prepared by fractionation with $(NH_4)_2SO_4$, precipitation with acetone, and dialysis. Traces of manganese or glycine greatly stimulated the activity of these preparations. In this respect the enzyme is similar to the alkaline phosphatase studied by Hove *et al.* (7).

Both spermatozoa and seminal fluid contained riboflavin as determined by the microbiological assay of Snell and Strong (8). A sample of spermatozoa separated from seminal fluid by centrifugation and washed once in 0.9 per cent saline contained 30 γ of riboflavin per gm. of dry tissue.[2] Flavin-containing enzymes are known to function in the hydrogen transport mechanisms

TABLE I

Dehydrogenase Activity of Spermatozoa As Measured by Thunberg Technique

Each Thunberg tube contained 1 cc. of 0.02 M substrate and 1 cc. of 0.0005 M methylene blue. 0.5 cc. of sperm suspension = 0.5 cc. of semen was placed in the bulb. After evacuation the tubes were placed in a water bath at 37° for 2 minutes before the sperm was mixed with the substrate-dye solution.

Sample	Reduction time					
	No added substrate	Succinate	Fumarate	Glucose	Lactate	Citrate
	min.	*min.*	*min.*	*min.*	*min.*	*min.*
A	9.5	5	9.5	75	32	
B	11	5	15	>90	45	20

of other tissues and it is probable that similar systems exist in spermatozoa.

Normal bull semen has been shown to contain from 2 to 8 mg. of ascorbic acid per 100 cc. (9). The metabolic function of this substance is not known. Ascorbic acid has been shown to promote the production of virile sperm in sterile bulls (9), but the *in vitro* addition of ascorbic acid to semen samples showing poor motility does not improve motility.[1]

Effect of Inhibitors—The effect of various inhibitors on the motility of sperm stored in yolk-buffer at 10° was first studied. Table II records results with one level of each inhibitor. These

[2] We wish to thank Miss Barbara Moore for the riboflavin assays.

observations have been repeated with sperm from several different
bulls. Lower concentrations of cyanide and iodoacetate allowed
motility but the storage time was decreased as will be shown
below. The effects of these inhibitors (except cyanide) on sperm
in the Ringer-phosphate-glucose medium at body temperature

TABLE II

Effect of Various Inhibitors on Motility of Sperm (Stored at 10°)

Medium, yolk-buffer plus	Motility rating					
	32 hrs.	55 hrs.	79 hrs.	103 hrs.	127 hrs.	172 hrs.
None.	5+	5+	4+	3+	2+	1+
0.001 M iodoacetate	Dead					
0.01 "fluoride . .	1+	F. M.*	Dead			
0.001 "cyanide	Dead					
0.01 "malonate	5+	4+	3+	2+	1+	F. M.
0.01 "selenate...... ..	5+	4+	4+	3+	2+	"
0.01 "selenite	Dead					
0.01 "arsenate . ..	5+	4+	2+	2+	1+	Dead
0.01 "arsenite..........	Dead					

* F. M. indicates that few progressively motile spermatozoa were
present.

TABLE III

Effect of Various Levels of Inhibitors on Aerobic Motility of Spermatozoa
(Incubated at 36°)

Medium, Ringer-phosphate-glucose plus	Motility			
	30 min.	85 min.	180 min.	255 min.
None...............	3+	2+	2+	1+
0.0005 M iodoacetate	3+	1+	Dead	
0.0025 " "	1+	Dead		
0.005 " "	Dead			
0.005 " fluoride	1+	1+	F. M.	Dead
0.01 " "	F. M.	Dead		

were analogous to those observed with yolk-buffer at 10°, and
therefore are not tabulated. Cyanide appeared to be less toxic
in the Ringer-phosphate medium than in yolk-buffer.

It was interesting to note that malonate, which is an inhibitor
of succinic dehydrogenase (the most active dehydrogenase in

sperm), had no effect on motility. Since the same concentration of malonate inhibited the "endogenous" oxygen uptake of washed sperm by 80 per cent, the lack of effect on motility was not due to the inability of malonate to penetrate the spermatozoa.

Iodoacetic acid was found to inhibit motility in proportion to the concentration used (Tables III and IV). Concentrations as

TABLE IV

Effect of Various Levels of Inhibitors on Anaerobic Lactic Acid Production and Motility of Spermatozoa (Incubated at 36° under N_2)

Medium, Ringer-phosphate-glucose plus	Motility		Lactic acid produced per cc. sperm suspension*	
	1 hr.	3 hrs	1 hr.	3 hrs.
			mg.	*mg.*
None	4+	2+	0.31	0.80
0.00025 M iodoacetate	2+	1+	0.00	0.00
0.0005 " " ...	2+	F. M.	0.00	0.00
0.003 " "	F. M.	Dead	0.00	0.00
0.005 " fluoride ...	Dead		0.00	0.00

* Sperm concentration = 500 million per cc.

TABLE V

Effect of Arsenate and Glutathione on Lactic Acid Production by Sperm

Medium, Ringer-phosphate-glucose plus	Lactic acid produced per cc. sperm suspension* in 90 min.
	mg.
None..	1.07
0.003 M GSH	1.03
0.001 " "	1.02
0.0002 " "	1.09
0.01 " arsenate	0.71
0.005 " "	1.04

* Sperm concentration = 1,050,000,000 per cc.

low as 0.00025 M completely inhibited lactic acid production; yet motility persisted for a considerable period of time. The data for aerobic lactic acid production were almost identical with the results shown in Table IV except that occasionally a small amount of lactic acid was produced in the presence of the lower concentrations of iodoacetate or in 0.005 M fluoride.

Other trials have shown that 0.002 and 0.001 M cyanide did not appreciably affect motility or glycolysis in the Ringer-phosphate medium.   Hydrogen peroxide is extremely toxic; a concentration of 0.01 M stopped all motility within 20 minutes.

Effect of Activators—Arsenate, which accelerates the breakdown of hexose diphosphate, did not increase lactic acid production by spermatozoa, as shown in Table V.   In fact concentrations of 0.01 M arsenate inhibited glycolysis.   Similarly, reduced gluta-thione (the coenzyme of glyoxalase) was without effect.

DISCUSSION

Ivanov showed that sperm remained motile in a medium con-taining sufficient cyanide to inhibit respiration almost completely (10).   In later studies (11) he found that motility was retained in a Ringer's solution containing sufficient monohaloacetic acid (0.002 to 0.004 M) to inhibit the lactic acid formation from glucose. From these observations he concluded that the sperm derives its energy by some process other than oxidation, and that motility does not depend on glycolysis.

The data presented here confirm Ivanov's observations.   In addition we have shown that spermatozoa retain motility for some time when glycolytic and oxidative mechanisms are blocked *simul-taneously* as shown by the experiments with sperm stored under N_2 in the presence of iodoacetate.   The mechanism by which sperma-tozoa obtain energy for motility under such conditions is being investigated.

Since malonate has no effect on motility, but does inhibit respira-tion, it seems that the succinic dehydrogenase of spermatozoa is not concerned with the maintenance of motility.   It may, how-ever, function in the oxidative removal of waste products.

The effect of selenium and arsenic salts on spermatozoa is similar to the effect observed by Potter and Elvehjem on the respiration of yeast (12) and on the succinoxidase system of homogenized chick kidney (13).   They too found selenite and arsenite to be highly toxic, while selenate and arsenate were relatively non-toxic.

The effect of cyanide on spermatozoa varies with the medium in which they are suspended.   A concentration of 0.001 M in-hibited the motility of sperm in yolk-buffer (Table II) but had little effect on motility or lactic acid production in the Ringer-

phosphate-glucose medium. From this observation it might appear that spermatozoa used the substrates of egg yolk solely by an oxidative process; however, when stored in yolk-buffer, spermatozoa remain motile equally well under N_2 or air.

Since arsenate did not increase the rate of glucose breakdown to lactic acid, it would appear that either hexose diphosphate was not an intermediary in the process or that its breakdown was so rapid under these experimental conditions that it was not a limiting factor in the rate of lactic acid production.

SUMMARY

Spermatozoa were found to contain succinic dehydrogenase and an alkaline phosphatase.

The study of the effects of inhibitors on sperm demonstrated that (1) low concentrations of iodoacetic acid completely inhibited the breakdown of glucose to lactic acid but did not directly affect motility, (2) selenate, arsenate, and malonate, at concentrations of 0.01 M, did not affect motility (the same levels of selenite, arsenite, fluoride, and H_2O_2 proved to be highly toxic), (3) 0.001 M cyanide inhibited the motility of sperm in yolk-buffer, but had little effect on motility or lactic acid production in a Ringer-phosphate-glucose medium.

Neither arsenate nor reduced glutathione had an appreciable influence on lactic acid production from glucose.

Although glycolytic and oxidative processes probably furnish energy for sperm motility under normal conditions, if both of these mechanisms are blocked spermatozoa still retain motility for considerable periods of time. It is evident that a process other than oxidation or glycolysis can furnish the energy for motility.

BIBLIOGRAPHY

1. Lardy, H. A., and Phillips, P. H., *Proc. Am. Soc. Animal Production*, 219 (1939).
2. Phillips, P. H., and Lardy, H. A., *J. Dairy Sc.*, **23**, 399 (1940).
3. Phillips, P. H., *J. Biol. Chem.*, **130**, 415 (1939).
4. Mendel, B., and Goldscheider, I., *Biochem. Z.*, **164**, 163 (1925).
5. Barker, S. B., *Am. J. Physiol.*, **129**, P305 (1940).
6. Lutwak-Mann, C., *Biochem. J.*, **34**, 517 (1940).
7. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **134**, 425 (1940)

8. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
9. Phillips, P. H., Lardy, H. A., Heizer, E. E., and Rupel, I. W., *J. Dairy Sc.*, **23**, 873 (1940).
10. Ivanov, E. E., *Z. Zùchtung, Reihe B*, **20**, 404 (1931).
11. Ivanov, I. I., *J. Physiol.*, U.S.S.R., **18**, 950 (1935). Ivanov, E. E., *Biochem. Z.*, **278**, 101 (1935).
12. Potter, V. R., and Elvehjem, C. A., *Biochem. J.*, **30**, 189 (1936).
13. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **117**, 341 (1937).

THE OXIDATION IN VITRO OF N-METHYLAMINO ACIDS BY KIDNEY AND LIVER

By PHILIP HANDLER,* FREDERICK BERNHEIM, AND J. RAYMOND KLEIN

(From the Departments of Physiology and Pharmacology and Biochemistry, Duke University School of Medicine, Durham, North Carolina)

The N-methyl derivatives of *dl*-methionine (1), *l*-cystine (2), *l*-tryptophane (3), *dl*-histidine (4), and *l*-phenylalanine (5) support the growth of albino rats on diets deficient in the corresponding amino acids. The N-methyl derivatives of *dl*-isoleucine, *dl*-leucine, *dl*-valine (5), *d*(+)-phenylalanine (5), and *d*(+)-tryptophane (6), the α-N-methyl and α-dimethyl derivatives of *dl*-lysine (7), and the N-dimethyl and N-ethyl derivatives of *dl*-phenylalanine (5) cannot replace the corresponding amino acids in the diet.

dl-N-Methylalanine is oxidized *in vitro* by kidney extracts to pyruvic acid and methylamine, while *dl*-N-dimethylalanine and *dl*-N-methyltyrosine are not (8). In the present work the oxidation of a series of N-methyl derivatives of amino acids by rat liver and kidney was studied. The special case of the oxidation of sarcosine will be considered in a subsequent publication.

EXPERIMENTAL

Oxidation of N-Methylamino Acids by Broken Cell Preparations of Rat Kidney[1]—The oxidation of the N-methylamino acids was tested with the rat kidney preparation previously described (10).

Oxygen uptakes were measured manometrically in the usual fashion in the Warburg apparatus. In the side arm of each vessel was placed 0.2 to 0.4 ml. of a solution of the substrate dissolved in 0.05 M phosphate buffer, pH 7.8, while the well contained 0.5 ml. of the kidney preparation and sufficient buffer to make a total volume of 2.0 ml. The insets contained alkali. The vessels were shaken in a bath at 38° for 2 hours. Typical data are presented in Table I. Data describing the oxidation of the corresponding amino acids are given for comparison. The

TABLE I

Oxidation of N-Methylamino Acids by Broken Cell Preparations of Rat Kidney

Substrate	Observed O_2 uptake	Theoretical O_2 uptake	Per cent of theoretical
	microliters	*microliters*	
dl-Methylleucine	20	156	13
dl-Leucine...	124	171	74
dl-Methylmethionine..	115	145	79
dl-Methionine..	125	150	84
dl-Methylphenylalanine... .. .	9	125	7
d(+)-Phenylalanine	227	272	84
dl-Methylvaline.	5	171	3
d(+)-Valine...	249	380	67
dl-Methyllysine....	6	140	4
dl-Lysine....	7	134	5
dl-Methyltryptophane....	2	102	2
dl-Methylalanine.	176	217	81
dl-Methylhistidine...	10	110	9
d(+)-Histidine.	86	213	40
l(−)-Histidine.	0	213	0

oxygen uptakes recorded in Table I are calculated by subtracting the uptake in a vessel containing tissue preparation and buffer from the uptake in the vessel containing tissue preparation, buffer, and the added substrate. The theoretical uptakes are calculated assuming the utilization of 1 atom of oxygen per mole of *d* isomer.

Table I shows that the N-methyl derivatives of *dl*-leucine, *dl*-methionine, and *dl*-alanine are oxidized by the kidney preparation. In the case of *dl*-N-methylleucine and *dl*-N-methylmethionine the amount of volatile base formed was about 85 per cent of that expected from the oxygen uptake.

The amount of volatile base was determined as follows: At the end of the period of oxidation the suspension in the respirometer flask was adjusted to pH 4.5 and heated for several minutes at 95–100°, and the proteins were removed by centrifugation. The solution was then made alkaline and the bases distilled at 40° into a known volume of standard acid. The residual acid was determined by back titration and the volatile base produced by the oxidation was calculated by subtracting the volatile base in the control vessel containing no added substrate from the volatile base in the vessel containing tissue preparation and added substrate.

The data for the oxidation of the other methylamino acids is equivocal. The oxygen uptakes, while positive, are of the order of the experimental error. Volatile base was obtained in the case of dl-N-methylhistidine but the amount was too small for an accurate estimation.

The rapid oxidation of dl-N-methylmethionine, illustrated in Fig. 1, permitted the identification of the keto acid formed. A purified preparation of the d-amino acid oxidase was obtained from the kidneys of four rats (11). This preparation was shaken for 150 minutes at 38° with 25 mg. of dl-N-methylmethionine in acetate buffer at pH 7.9. The mixture was adjusted to pH 5 and heated in a bath at 80° for several minutes, and the proteins were removed by centrifugation. The supernatant fluid was concentrated *in vacuo* and made alkaline with potassium carbonate. After 75 mg. of phenylhydrazine hydrochloride were added, the solution was allowed to stand 3 hours at room temperature and then acidified to pH 3 and placed in the refrigerator. After 48 hours the solids which had appeared were filtered and recrystallized from 60 per cent ethyl alcohol. The yield was 4.1 mg. of material which melted at 134°. The phenylhydrazone of α-keto-γ-methiolbutyric acid melts at 134–135° (12).

That the enzyme catalyzing the oxidation of the N-methyl derivatives of dl-methionine, dl-leucine, and dl-alanine was the d-amino acid oxidase was demonstrated in the following manner: A mixture of 4 mg. of a preparation of the protein component of the d-amino acid oxidase and 2.5 γ of flavin-adenine dinucleotide in 2.5 ml. of 0.05 M pyrophosphate buffer, pH 8.3, was incubated with 1 mg. of each methylamino acid at 37.5°, and the oxygen

uptake and the carbon dioxide production measured in the usual fashion. The protein was prepared from pig kidney and the flavin-adenine dinucleotide from yeast (13).

dl-N-Methylmethionine and *dl*-N-methylalanine were oxidized with an oxygen uptake of 1 mole of oxygen and the production of 1 mole of CO_2 per mole of d isomer. The extent of the oxidation was 90 to 100 per cent of the theoretical in 85 minutes, at which time the reaction had practically stopped. In the same time *dl*-N-methylleucine was oxidized 17 per cent of the theoretical amount and *dl*-N-methylhistidine 5 per cent. The other methylamino acids were not oxidized in 85 minutes.
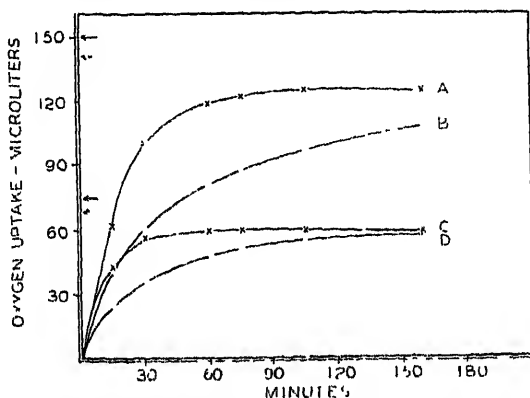
Fig. 1. The oxidation of *dl*-N-methylmethionine by broken cell preparations of rat kidney. Curves A and C represent the oxidation of 4 and 2 mg. respectively of *dl*-methionine. Curves B and D show the oxidation of 4 and 2 mg. respectively of *dl*-N-methylmethionine. The solid arrows represent the theoretical O_2 uptakes for Curves A and C; the dotted arrows represent the theoretical O_2 uptakes for Curves B and D.

Oxidation of N-Methylamino Acids by Broken Cell Preparations of Rat Liver—The oxidation of N-methylamino acids by rat liver was studied in the same manner as that described for kidney. The results obtained in this manner are summarized in Table II.

It will be seen that the same conclusions may be drawn from the data in Table II as from those in Table I. The failure of both types of preparation to oxidize $d(-)$-lysine is in accord with the observations of Felix and Zorn (14). In addition, these data indicate that liver can oxidize *dl*-N-methylhistidine. Moreover, neither preparation is capable of oxidizing $l(-)$-histidine.

Volatile base determinations, performed in the manner described, indicated that the oxidation of *dl*-N-methylhistidine was an oxidative deamination. The volatile base produced by the incubation of both *dl*-N-methylhistidine and *d*(+)-histidine was within 85 per cent of the theoretical amount calculated from the oxygen uptake. Since the *d*-amino acid oxidase activity of kidney is greater than is that of liver, it does not seem probable that the oxidation of the methylhistidine is catalyzed by this system. This is further indicated by the experiments with the reconstructed *d*-amino acid oxidase system described above.

TABLE II

Oxidation of N-Methylamino Acids by Broken Cell Preparations of Rat Liver

Substrate	Observed O₂ uptake	Theoretical O₂ uptake	Per cent of theoretical
	microliters	*microliters*	
dl-Methylleucine.................	22	156	14
dl-Methylmethionine..............	60	145	42
dl-Methylvaline........	4	171	3
dl-Methylphenylalanine..	11	125	8
dl-Methyltryptophane.............	6	102	6
dl-Methyllysine...................	0	140	0
dl-Lysine.................	4	67	6
dl-Methylalanine..........	113	217	52
dl-Methylhistidine................	58	110	53
d(+)-Histidine..., ...	57	120	48
l(−)-Histidine...................	0	120	0

Oxidation of Methylamino Acids by Liver and Kidney Slices— An attempt was made to study the oxidation of methylamino acids by liver and kidney slices. However, extremely ambiguous results were obtained. Only *dl*-N-methylphenylalanine and *dl*-N-methylmethionine produced extra volatile base and none of the compounds increased the rate of respiration of either liver or kidney slices. The production of methylamine (measured by a micro-Kjeldahl determination of the volatile bases after the incubation period) from the substituted phenylalanine by kidney slices was of the order of 1.5 micromoles per gm. of kidney (wet weight) in 2 hours. No methylamine production from *dl*-methylphenylalanine was observed with liver slices. The *dl*-N-methylmethionine was oxidized at a rate of approximately 10 micro-

moles per gm. of kidney in 2 hours. The rate of oxidation of this compound by liver slices was about one-third of that by kidney slices.

DISCUSSION

It has been suggested that, in order to replace l-amino acids in the diet, the corresponding methylamino and d-amino acids may be oxidized to the α-keto acids and then reaminated to the l-amino acids. However, the ability of the tissues of an animal to deaminate oxidatively a d-amino acid *in vitro* does not necessarily indicate that the d form can replace the l isomer in the diet; *e.g.*, the d forms of valine, leucine, isoleucine (12), and dl-N-methylleucine are oxidized by rat liver and kidney preparations to the corresponding α-keto acids but cannot replace the l-amino acids in the diet (15). The inability of the tissues of an animal to oxidize a d-methylamino or d-amino acid may be expected to preclude the possibility of the utilization of this compound. From this standpoint the inability of the broken cell preparations of liver and kidney used in this study to oxidize dl-N-methyltryptophane, dl-N-methylphenylalanine, dl-N-methylvaline, and dl-lysine at an appreciable rate is in accord with the inability of the d forms of these compounds to replace the corresponding l-amino acids in the diet of the albino rat. The extremely small rate of oxidation of l-amino and l-methylamino acids does not permit any correlation between *in vivo* and *in vitro* experiments. It would be interesting, in the light of the present observations, to know whether the d forms of N-methylmethionine and N-methylhistidine, which have been fed only as racemic mixtures, can support growth.

SUMMARY

1. Broken cell preparations of rat kidney oxidatively demethylaminate the N-methyl derivatives of dl-methionine, dl-alanine, and dl-leucine.
2. The d-amino acid oxidase is responsible for these oxidations.
3. Broken cell preparations of rat liver oxidize these methylamino acids and also dl-N-methylhistidine.
4. Neither liver nor kidney preparations oxidize the N-methyl

derivatives of *dl*-phenylalanine, *dl*-tryptophane, *dl*-valine, *dl*-lysine, or *l*(−)-histidine at an appreciable rate.

BIBLIOGRAPHY

1. Patterson, W. I., Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **116**, 277 (1936).
2. Kies, M. W., Dyer, H. M., Wood, J. L., and du Vigneaud, V., *J. Biol Chem.*, **128**, 207 (1939).
3. Gordon, W. G., and Jackson, R. W., *J. Biol. Chem.*, **110**, 151 (1935).
4. Fishman, J. B., and White, A., *J. Biol. Chem.*, **113**, 175 (1936).
5. Carter, H. E., and Handler, P., *Proc. Soc. Exp. Biol. and Med.*, **41**, 347 (1939).
6. Gordon, W. G., *J. Biol. Chem.*, **129**, 309 (1939).
7. Gordon, W. G., *J. Biol. Chem.*, **127**, 487 (1939).
8. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 114, 141 (1936).
9. Handler, P., Thesis, University of Illinois (1939).
10. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **106**, 79 (1934).
11. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **109**, 131 (1935).
12. Bernheim, F., Bernheim, M. L. C., and Gillaspie, A. G., *J. Biol. Chem.*, **114**, 657 (1936).
13. Warburg, O., and Christian, W., *Biochem Z.*, **298**, 150, 369 (1938).
14. Felix, K., and Zorn, K., *Z. physiol. Chem.*, **258**, 16 (1939).
15. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).

THE OXIDATIVE DEMETHYLATION OF SARCOSINE
TO GLYCINE

By PHILIP HANDLER,* MARY L. C. BERNHEIM, and J. RAYMOND
KLEIN

*(From the Departments of Physiology and Pharmacology and Biochemistry,
Duke University School of Medicine, Durham, North Carolina)*

The coupled feeding of sarcosine (N-methylglycine) and ben-
zoic acid, like the coupled feeding of glycine and benzoic acid,
results in the excretion of hippuric acid at a rate greater than that
when benzoic acid is fed alone (1). When sarcosine containing
an excess of N^{15} is fed to rats, the isotope content of the amino
acids of the tissues is almost identical with that of animals fed
glycine containing N^{15}. The isotope concentration in the glycine
from the tissue proteins is high, while that of the other amino
acids is much lower (2). These findings have indicated that sar-
cosine can be converted to glycine by direct demethylation without
deamination.

In the present work it has been shown that broken cell prepara-
tions of the livers of several species, as well as surviving liver slices,
can perform this conversion. The demethylation is an oxidative
process in which 1 mole of sarcosine reacts with 1 atom of oxygen
with the production of 1 mole of glycine and 1 mole of formal-
dehyde. The mechanism is shown in the accompanying formula.
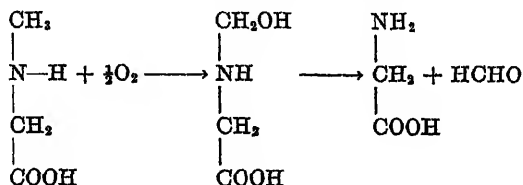
$$
\begin{array}{ccc}
CH_3 & CH_2OH & NH_2 \\
| & | & | \\
N-H + \tfrac{1}{2}O_2 \longrightarrow NH & \longrightarrow CH_2 + HCHO \\
| & | & | \\
CH_2 & CH_2 & COOH \\
| & | & \\
COOH & COOH &
\end{array}
$$

Oxidation of Sarcosine by Broken Cell Preparations—The sarcosine used in this study was obtained from the Eastman Kodak Company.

The tissue was prepared by grinding it with an equal volume of 0.05 M phosphate buffer, pH 7.8. The suspensions were filtered through muslin and used directly. Oxygen uptakes were measured in the usual fashion in the Warburg apparatus. In the side arm of each vessel was placed 0.1 to 0.5 ml. of a solution of the substrate dissolved in 0.05 M phosphate buffer, pH 7.8, while the well contained 0.25 to 1.0 ml. of the tissue preparation and sufficient buffer to make a total volume of 2.0 ml. The insets contained alkali. The vessels were shaken in a bath at 38°. In each instance a suitable control vessel containing tissue preparation and buffer but no added substrate was shaken simultaneously. All oxygen uptakes referred to hereafter represent the difference between the oxygen uptakes in the vessels containing added substrate and the control vessels.

The oxidation of sarcosine is quite rapid; 0.5 mg. is completely oxidized in 75 minutes by 1.0 ml. of suspension. The *sarcosine oxidase* activity was found to be confined to the insoluble proteins of the liver suspension. With fresh rat liver protein preparations which had twice been washed with 0.05 M phosphate buffer, the oxidation was accelerated; the oxidation of 0.5 mg. of sarcosine was complete in 45 minutes. In Fig. 1 are shown the comparative rates of oxidation of various amounts of sarcosine by washed rat liver protein preparations.

The tissues of the rat, rabbit, and guinea pig were examined for their ability to oxidize sarcosine. The livers of each species are approximately equally potent in this respect. Neither kidney nor muscle preparations of any species appear to contain any *sarcosine oxidase*. The nature of the end-products of the reaction was established in the following manner. 4 mg. of sarcosine were shaken for 150 minutes at 38° with 4.0 ml. of a washed rat liver protein suspension and 12.0 ml. of 0.05 M phosphate buffer, pH 7.8, in an open vessel. A control solution containing no sarcosine was incubated simultaneously. The course of the reaction was followed in the Warburg apparatus, with 2.0 ml. aliquots of each solution. To each solution were then added 4.0 ml. of 20 per cent

trichloroacetic acid solution and the proteins then removed by centrifugation.

Equal volumes of each solution were then made alkaline and the volatile bases distilled *in vacuo* into a standard amount of 0.02 N HCl. The amount of volatile base was determined by back titration. The titers in each case were identical. The reaction, therefore, is not an oxidative deamination, since neither ammonia nor methylamine is produced.
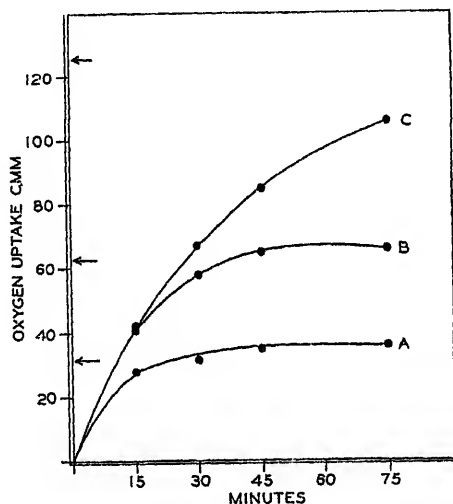
Fig. 1. The oxygen uptake of 0.25 mg. (Curve A), 0.5 mg. (Curve B), and 1.0 mg. (Curve C) of sarcosine in the presence of 0.5 ml. of the standard washed rat liver protein preparation, pH 7.8, at 38°. The theoretical uptakes, assuming 1 atom of oxygen per mole of sarcosine, are indicated by the arrows.

Another sample of each solution was neutralized and shaken twice with permutit. Qualitatively the presence of glycine in the solution of oxidized sarcosine was shown by the sodium hypochlorite-phenol test (3). The same test performed on the control solution was negative. However, since this test is also given by sarcosine, ammonia, and methylamine, it could not be considered conclusive. Van Slyke amino nitrogen determinations were performed in duplicate on 2.0 ml. aliquots of each solution. The solution of oxidized sarcosine was found to contain 0.0632

mg. of amino nitrogen, equivalent to 0.34 mg. of glycine per 2.0 ml., in excess of that found in the control solution.   The theoretical glycine content calculated from the oxidation of the original sarcosine was 0.336 mg. per 2.0 ml.   Since no methylamine or ammonia had been formed during the oxidation, and if they had been formed would have been removed by the permutit, the sarcosine must have been converted to glycine.

Aliquots of the two trichloroacetic acid filtrates were also used to demonstrate the presence of formaldehyde.   Strongly positive tests were obtained in the solution of oxidized sarcosine by the phenylhydrazine-ferric chloride test (4), and by Rimini's phenylhydrazine-nitroprusside test (4).   The control solution gave negative results with both reagents.   The indole test (5) for glyoxylic acid was negative in both solutions.

When methyl alcohol was incubated with washed rat liver proteins under the same conditions used in the oxidation of sarcosine, it was not oxidized at an appreciable rate.   This rules out the possibility that the sarcosine is hydrolyzed to glycine and methyl alcohol and the latter then oxidized.

Washed liver protein preparations were used to study the effect of pH on the activity of the enzyme system.   The optimal pH for the activity of sarcosine oxidase is about 7.8.   However, the enzyme retains some activity at pH 6.0 and 8.1.   This is illustrated in Fig. 2.

The rate of oxidation of sarcosine also varies with the concentration of the enzyme.   In Fig. 3 is shown the oxidation of 0.5 mg. of sarcosine in the presence of 0.25 to 1.5 ml. of the standard washed rat liver protein suspension.   It will be seen that the rate increases as the enzyme concentration is raised up to 1.0 ml.   Further increases in the concentration have little effect.

The rate of oxidation of sarcosine is not affected by the presence of equimolar amounts of glycine or formaldehyde.   Larger quantities of formaldehyde but not glycine completely inhibit the reaction.   The enzyme system is heat-labile; it is destroyed by heating for 3 minutes in a boiling water bath.   The liver proteins rapidly lose their activity, even when stored in the refrigerator; 24 hour-old preparations still retain some of their potency but the rate of sarcosine oxidation is small.   The addition of adenylic acid, flavin-adenine dinucleotide, di- or triphosphopyridine nucleo-

tides, or boiled liver extract does not restore this activity nor do they increase the rate of oxidation of sarcosine by fresh preparations. When a washed liver preparation was incubated with sarcosine in the presence of methylene blue in a Thunberg tube, the methylene blue was rapidly reduced.
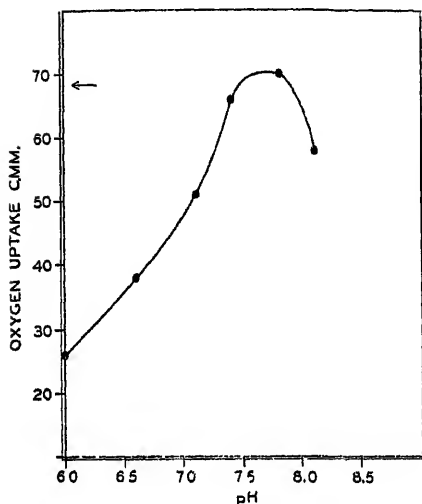
FIG. 2.   FIG. 3.

FIG. 2. The effect of pH on sarcosine oxidase. Each point represents the oxygen uptake in 30 minutes of 0.5 mg. of sarcosine in the presence of 0.5 ml. of the standard washed rat liver protein preparation at the pH indicated. Bath temperature 38°. The theoretical uptake, assuming 1 atom of oxygen per mole of sarcosine, is indicated by the arrow.
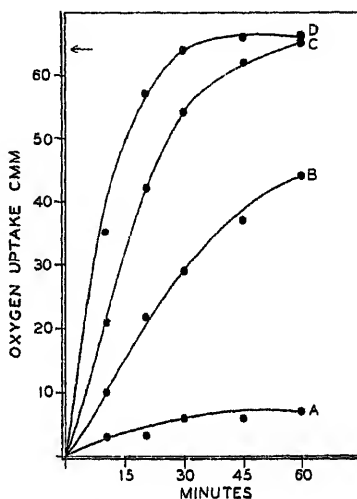
FIG. 3. The effect of varying the concentration of sarcosine oxidase. The curves represent the oxygen uptake of 0.5 mg. of sarcosine at pH 7.8 in the presence of 0.25 ml. (Curve A), 0.5 ml. (Curve B), 1.0 ml. (Curve C), and 1.5 ml. (Curve D) of the standard washed rat liver protein preparation at 38°. The theoretical uptake, assuming 1 atom of oxygen per mole of sarcosine, is indicated by the arrow.

Specificity of Enzyme—It has been shown that the oxidation of several N-methylamino acids by liver preparations is accompanied by demethylamination (6). It, therefore, seemed desirable to examine further the specificity of sarcosine oxidase. For this purpose N-ethylglycine was prepared by heating a solution of 3 gm. of monochloroacetic acid in 15 ml. of 33 per cent ethylamine

solution at 100° for 12 hours.   After the solution was concentrated in the presence of a large excess of barium hydroxide and the latter was removed with sulfuric acid, the solution was further concentrated in the presence of an excess of hydrochloric acid to a thin syrup.   On the addition of acetone the N-ethylglycine hydrochloride readily crystallized.   Recrystallization was effected by dissolving the material in a small amount of water, filtering through norit, and adding acetone slowly.

$C_4H_{10}O_2NCl$.   Calculated, N 10.07; found, N 9.98
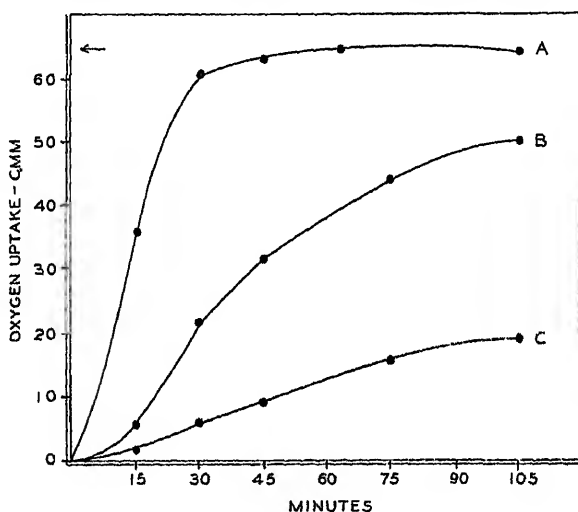
Fig. 4. The oxygen uptake of 0.056 millimole of sarcosine (Curve A), N-dimethylglycine (Curve B), and N-ethylglycine (Curve C) in the presence of 1.0 ml. of standard washed rat liver protein preparation, pH 7.8, at 38°.   The theoretical uptake, assuming 1 atom of oxygen per mole of substrate, is indicated by the arrow.

In the same manner N-dimethylglycine hydrochloride was prepared from dimethylamine and monochloroacetic acid, while N-methyl-β-alanine hydrochloride was prepared from β-bromo-propionic acid and methylamine, the bromide having been removed with silver oxide.

N-Dimethylglycine Hydrochloride—
        $C_4H_{10}O_2NCl$.   Calculated, N 10.07; found, N 10.11
    N-Methyl-β-Alanine Hydrochloride—
        $C_4H_{10}O_2NCl$.   Calculated, N 10.07; found, N 9.91

Glycine, N-ethylglycine, N-dimethylglycine, betaine, and N-methyl-β-alanine were incubated with rat liver preparations of sarcosine oxidase. Of these compounds only N-ethyl- and N-dimethylglycine were oxidized at an appreciable rate. The rate of oxidation of the latter was greater than the rate of oxidation of the former. This is shown in Fig. 4. The solution of oxidized N-dimethylglycine gave positive tests for the presence of formaldehyde, while the formation of glycine was indicated by Van Slyke amino nitrogen determinations. The amount of glycine was approximately one-half of the theoretical amount calculated from the oxygen uptakes, assuming 2 atoms of oxygen per mole of dimethylglycine. Since the total oxidation was incomplete, it is reasonable to assume that the solution analyzed contained a mixture of dimethylglycine, sarcosine, and glycine.

Oxidation of Sarcosine by Liver Slices—The oxidation of sarcosine by surviving rat liver slices is quite slow. The extra oxygen uptake of liver slices in the presence of 0.0038, 0.0075, and 0.015 M sarcosine was 0, 15, and 25 microliters per 30 minutes per 100 mg. of wet tissue. The amino nitrogen formed in the presence of 0.015 M sarcosine was 65 microliters per 30 minutes per 100 mg. of wet tissue. Thus the extra oxygen uptake in 3.0 ml. of solution containing 0.045 millimole of sarcosine was 0.0011 millimole of oxygen, while 0.0029 millimole of amino nitrogen was formed.

DISCUSSION

The *in vivo* experiments of Abbott and Lewis (1) and Bloch and Schoenheimer (2) have suggested that sarcosine may be converted to glycine by direct demethylation. The present work demonstrates that there exists, in the livers of those species examined, an enzyme system capable of performing this demethylation. It is postulated that the N-methyl group is oxidized to an N-methylol group. Under the conditions of these experiments such an N-methylol group would dissociate, yielding glycine and formaldehyde. It cannot be stated whether the same dissociation occurs *in vivo* or whether the methylol group is further oxidized while attached to the nitrogen atom. There is no adequate evidence concerning the metabolism of small amounts of formaldehyde although the presence of small amounts of formic acid in blood has been suggested by Orskov (7) and in urine by Dakin *et al.* (8).

This direct demethylation of sarcosine does not necessarily indicate that other N-methylamino acids are metabolized in the same fashion. Rat liver and kidney preparations identical with those used in this study have been shown (6) to be unable to oxidize the N-methyl derivatives of dl-phenylalanine, dl-tryptophane, and dl-valine. The oxidation of the N-methyl derivatives of dl-methionine and dl-leucine by these preparations has been shown to be due to the presence of d-amino acid oxidase, and the oxidation of dl-N-methylhistidine to be an oxidative demethylamination. Except for the fact that several of these compounds have been found capable of replacing the unsubstituted corresponding amino acids in the diet of the white rat (9), nothing is known of the intermediary metabolism of the l forms of these methylamino acids. However, it can be stated that they are not oxidatively demethylated *in vitro* by sarcosine oxidase.

SUMMARY

Broken cell preparations of rat, rabbit, and guinea pig liver but not kidney or muscle oxidatively demethylate sarcosine to glycine. 1 mole of sarcosine reacts with 1 atom of oxygen with the production of 1 mole of glycine and 1 mole of formaldehyde. These preparations also oxidize N-ethyl- and N-dimethylglycine but not betaine or N-methyl-β-alanine. The demethylation can also be accomplished by liver slices although the rate of reaction is quite small.

BIBLIOGRAPHY

1. Abbott, L. D., Jr., and Lewis, H. B., *J. Biol. Chem.*, **131**, 479 (1939).
2. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 99 (1940).
3. Fürth, O., Friedrich, A., and Scholl, R., *Biochem. Z.*, **240**, 50 (1931).
4. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 4th edition, 437 (1935).
5. Spiro, K., *Z. anal. Chem.*, **46**, 270 (1907).
6. Handler, P., Bernheim, F., and Klein, J. R., *J. Biol. Chem.*, **138**, 203 (1941).
7. Orskov, S. L., *Skand. Arch. Physiol.*, **63**, 255 (1932).
8. Dakin, H. D., Janney, N. W., and Wakeman, A. J., *J. Biol. Chem.*, **14**, 341 (1913).
9. Lewis, H. B., and Garner, R. L., in Luck, J. M., Annual review of biochemistry, Stanford University, **9**, 287 (1940).

THE RELATION OF COPPER TO CYTOCHROME OXIDASE AND HEMATOPOIETIC ACTIVITY OF THE BONE MARROW OF RATS*

By M. O. SCHULTZE

(*From the Department of Chemistry, University of Pittsburgh, Pittsburgh*)

Recent studies have shown that copper deficiency of the rat leads to a great reduction of the cytochrome oxidase activity of the liver and the heart (1). Considering the fact that in extra-uterine life the bone marrow is the main center of hematopoietic activity and that copper is necessary for normal hematopoiesis, it was of interest to determine cytochrome oxidase activity of the bone marrow (*a*) during copper deficiency and recovery from it, and (*b*) in other conditions of diminished or accelerated hematopoietic activity. The results of such a study are reported here.

EXPERIMENTAL

Animals—Iron deficiency and copper deficiency in rats were produced as described previously (1). The milk, after H_2S treatment and concentration *in vacuo*, was fortified with sufficient thiamine chloride to insure a daily intake of at least 20 γ of this vitamin. All metallic supplements were fed with a small quantity of milk. The rats in Groups 10 to 13 received the stock diet of Purina Dog Chow. Group 11 was subjected to severe hemorrhage by heart puncture under light ether anesthesia by which 20 to 30 per cent of the assumed blood volume (7 per cent of the body weight) was removed.

The animals in Group 12 were exposed to low oxygen tension by keeping them in pairs in a large desiccator evacuated to 420

to 430 mm. of Hg.   A constant flow of air was drawn through the desiccator and the vacuum was regulated by a capillary stop-cock.

Cytochrome Oxidase Determination—The rats were decapitated and the long bones (humeri, femora, tibiae) were dissected.   The marrow was removed from the bones after they were split longitudinally with a sharp scalpel.   It was then suspended in redistilled water and homogenized (2).   An aliquot of the marrow suspension was dried to constant weight at 105° and the remainder was used for determination of cytochrome oxidase activity.   This determination was carried out manometrically as described earlier (1).   Each of two duplicate Warburg vessels contained 0.75 ml. of marrow suspension, 0.1 ml. of cytochrome c (1×10^{-7} M), 0.2 ml. of neutral semicarbazide (2.8×10^{-5} M), and 1.5 ml. of M/15 phosphate buffer, pH 7.2.   The side arm contained 0.25 ml. of hydroquinone (5×10^{-5} M) and the center cup contained 0.2 ml. of 20 per cent KOH.   A control vessel without added cytochrome c was always run in parallel.   The determinations were made at 37.2° and for a 25 minute period after equilibration and closing of the stop-cocks.   The cytochrome oxidase activity was calculated from the oxygen uptake of the system (minus the blank) during a 20 minute interval (between the 5 and 25 minute periods) and expressed as $Q_{oxidase} =$ (c.mm. of oxygen absorbed)/ (hour $\times$ mg. of dry marrow).

DISCUSSION

Table I summarizes the results.   The bone marrow of young rats in full hematopoietic activity has an oxidase quotient of 30 to 35 (Group 1).   Mature rats which need to make erythroid elements only for maintenance of the normal blood picture have a distinctly lower cytochrome oxidase activity of the bone marrow (Groups 2 and 10).   In severe anemia of iron deficiency the cytochrome oxidase activity of the bone marrow corresponds to that found in rats with full hematopoietic activity (Group 3).   It appears that under these conditions the bone marrow is prepared for hematopoietic activity and that it lacks only iron for incorporation into the hemoglobin molecule and perhaps for the synthesis of other heme compounds.   In copper deficiency, however, the cytochrome oxidase activity of the bone marrow is very low

(Group 4). In this case the hematopoietic failure is associated with an enzyme deficiency in the bone marrow. This is emphasized by the fact that in early copper deficiency, when the development of anemia is accelerated as a result of rapid growth and increasing blood volume, the bone marrow is already deficient

TABLE I

Cytochrome Oxidase of Bone Marrow of Rats

Group No.	Diet	Condition of rats and treatment	No. of rats	Hb	$Q_{oxidase}$
				per cent	
1	Milk + Fe + Cu + Mn	Young, growing	10	9–12	33.4 ± 4.1
2	Same	Mature	8	12–14	19.8 ± 1.9
3	Milk + Cu + Mn	Fe-deficient	15	3– 5	31.9 ± 5.2
4	Milk + Fe + Mn	Cu-deficient	21	2– 4	5.6 ± 3.6
5	Same	Early Cu deficiency; 0–2 wks. after weaning	10	4– 6	10.4 ± 3.3
6	"	Cu-deficient, fed 0.1 mg. Cu 24 hrs. previously	11	2– 4	29.0 ± 3.5
7	"	Cu-deficient, fed 0.1 mg. Cu per day 2 days	10	3– 4	29.6 ± 3 0
8	"	Cu-deficient, fed 0.1 mg. Cu per day 5 days	10	5– 6	34.2 ± 7.0
9	"	Cu-deficient, fed 2 mg. Co during previous 3½ days	10	2– 4	6.3 ± 1.3
10	Stock diet	Mature	12	12–14	18.6 ± 4.6
11	Same	" hemorrhage 48 hrs. previously	10	8–10	34.1 ± 5.2
12	"	Mature; kept under low O_2 tension 42 hrs. previously	10	12–14	31.5 ± 3.6
13	"	Mature; fed 2 mg. Co during previous 3½ days	10	12–14	29.7 ± 3.7

in cytochrome oxidase activity (Group 5). Most striking, however, is the immediate response of the copper-deficient bone marrow to copper therapy. 24 hours after 0.1 mg. of copper was fed to severely deficient rats, the bone marrow had attained a cytochrome oxidase activity similar to that found in marrows with

full hematopoietic activity (Group 6).   This response is practically maximal, since the cytochrome oxidase activity of the bone marrow does not increase appreciably when copper therapy is prolonged (Groups 7 and 8).   It may be well to point out that 24 hours after the initial copper therapy a reticulocyte response in the circulating blood is hardly noticeable (3).   The results presented here suggest that the cytochrome oxidase of the bone marrow must be brought to or maintained at a certain level (as yet quantitatively undetermined) before hematopoiesis can proceed successfully.   It is clear that for this process copper must be available to the animal.   The entrance of copper into the bone marrow within 24 hours following copper therapy has been demonstrated by means of radioactive copper (4).   The observations reported here on the effect of copper on the cytochrome oxidase activity of the bone marrow are in harmony with the results previously found with liver and heart (1).

If cytochrome oxidase activity of the bone marrow is a prerequisite for and closely associated with hematopoietic activity, it should be possible to observe increased cytochrome oxidase activity in the bone marrow of mature, normal rats which have been subjected to hematopoietic stimuli for very short periods. Severe hemorrhage, low oxygen tension, and feeding of cobalt salts can be used as powerful stimuli for hematopoietic activity of the bone marrow.   Under all three conditions there is a rapid increase of the cytochrome oxidase activity of the bone marrow to a level associated with rapid blood formation (Groups 11 to 13). Cobalt alone cannot elicit such a response, since copper-deficient rats do not respond to the stimulus of cobalt (Group 9).   Underhill et al. (5) have shown that copper-deficient rats fail to develop cobalt polycythemia.   The effect of copper on cytochrome oxidase activity is therefore specific.   Normal mature rats contain or have access to sufficient copper to permit the formation of increased cytochrome oxidase activity in the bone marrow under the influence of hematopoietic stimuli.

The observations reported here demonstrate clearly that (1) a high cytochrome oxidase activity of the bone marrow is intimately associated with hematopoiesis, provided the latter process is not interfered with by other deficiencies, and (2) copper is essential for the formation and the maintenance of cytochrome oxidase activity of the bone marrow.

It appears, therefore, that the effect of copper on blood formation can be accounted for, at least in part, by its ability to provide for adequate enzymatic and metabolic activity at the site of hematopoiesis. In view of the low oxygen tension in the erythrogenic centers and their great synthetic activity, such a concept does not appear unreasonable. It has been suggested that the copper content of the blood may be of functional significance in relation to blood formation (6, 7). Further work will be necessary to determine whether the copper content of the blood bears any relation to the cytochrome oxidase activity of the bone marrow.

The chemical nature of the compound (or compounds) responsible for cytochrome oxidase activity of animal tissues is not yet fully understood. A metalloporphyrin with a hemochromogen spectrum is apparently involved in the cytochrome oxidase activity of heart muscle preparations (8). It has been suggested that copper may be a structural component of cytochrome oxidase (9). If this were true, it would provide, together with the observations presented here, a rational explanation for the effect of copper on hematopoiesis. Until isolation permits an analysis of cytochrome oxidase, such reasoning remains largely speculative. If, on the other hand, cytochrome oxidase is an iron-porphyrin compound, its synthesis, like that of hemoglobin and of catalase (10), depends on the presence of copper. In that case the effect of copper on hematopoiesis would be dual, first on cytochrome oxidase activity of the bone marrow and second on the synthesis of hemoglobin.

SUMMARY

1. Copper deficiency causes a great decrease of the cytochrome oxidase activity of the bone marrow of rats.

2. Copper therapy of deficient rats initiates an immediate increase in cytochrome oxidase activity of the bone marrow. Within 24 hours maximum activity is approached.

3. Iron, manganese, or cobalt does not affect cytochrome oxidase activity in the absence of copper.

4. Normal mature rats respond to hematopoietic stimuli (hemorrhage, low oxygen tension, feeding of cobalt) by a rapid increase of cytochrome oxidase activity of the bone marrow.

5. There exists a close relation between the cytochrome oxidase activity of the bone marrow and its ability to form hemoglobin and erythrocytes.

BIBLIOGRAPHY

1. Schultze, M. O., *J. Biol. Chem.*, **129**, 729 (1939).
2. Potter, V. R , and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
3. Schultze, M. O., and Elvehjem, C. A., *J. Biol. Chem.*, **102**, 357 (1933).
4. Schultze, M. O., and Simmons, S. J., Abstracts of the Conference on Nuclear Physics, Boston, October 28 to November 2 (1940).
5. Underhill, F. A., Orten, J. M , and Lewis, R. C., *J. Biol. Chem.*, **91**, 13 (1931).
6. Schultze, M. O., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **116**, 107 (1936).
7. Schultze, M. O., *Physiol. Rev.*, **20**, 37 (1940).
8. Keilin, D. R., and Hartree, E. F., *Proc. Roy. Soc. London, Series B.*, **127**, 167 (1939).
9. Keilin, D. R., and Hartree, E. F., *Nature*, **141**, 870 (1938).
10. Schultze, M. O., and Kuiken, K. A., *J. Biol. Chem.*, **137**, 727 (1941)

STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

XII. THE SYNTHESIS OF N-ACETYL-S-*p*-BROMOBENZYL-*l*-CYS-TEINE IN THE RAT FROM *p*-BROMOBENZYL BROMIDE, S-*p*-BROMOBENZYL-*l*-CYSTEINE, AND S-*p*-BROMOBENZYLGLUTATHIONE

By JAKOB A. STEKOL

(*From the Department of Chemistry, Fordham University, New York*)

The synthesis of mercapturic acids *in vivo* from various aromatic compounds no longer can be considered an isolated instance of a detoxication reaction involving cysteine. Within the past few years, it has been demonstrated that six substances, rather dissimilar in structure and properties, are partially converted *in vivo* to the corresponding mercapturic acids. These substances are the three monohalogenated benzenes, naphthalene, anthracene, and benzyl chloride. Several workers have demonstrated that the synthesis of these mercapturic acids takes place in dogs (2, 5, 8, 14), rabbits (1, 3, 4, 6, 7, 14), swine (10), cats (17), rats (4, 6, 12, 14, 18), and mice (9).

The present report deals with the synthesis of a new mercapturic acid in the rat from *p*-bromobenzyl bromide, *p*-bromobenzyl-cysteine, and *p*-bromobenzylglutathione.

EXPERIMENTAL

Preparation of Compounds

S-p-Bromobenzyl-l-Cysteine—15.6 gm. of *l*-cysteine hydrochloride were dissolved in 100 cc. of 3.0 N NaOH. 25.1 gm. of *p*-bromobenzyl bromide[1] (saturated solution in 95 per cent ethanol) were added dropwise with shaking to the solution. After all of the bromide had been added, the solution was shaken vigorously

---

[1] *p*-Bromobenzyl bromide is extremely irritating to the eyes and mucous membranes.

for about 10 minutes, and then extracted with two 100 cc. portions of ether. To the aqueous layer, concentrated HCl was added to bring the pH of the solution to 6.0. After the solution had been cooled in the refrigerator for 3 hours, the precipitate was removed by filtration, washed with cold water, then recrystallized three times from 2 liters of boiling water, and dried *in vacuo* over H_2SO_4. The yield was 30 gm., or about 89 per cent of the theoretical amount.

	C	H	N	S	M.p.*
Found	41.22	4.40	4.78	11 12	213–214
$BrC_{10}H_{12}O_2NS$. Calculated..	41.25	4.48	4.81	11 00	

* All melting points are corrected.

1.0 per cent of the substance in N NaOH had a specific rotation of $[\alpha]_D^{24} = +23°$.

N-Acetyl-S-p-Bromobenzyl-l-Cysteine—1.0 gm. of S-*p*-bromo-benzyl-*l*-cysteine was dissolved in 30 cc. of 2.5 N NaOH, and 2 cc. of acetic anhydride were added to the solution dropwise with shaking, the flask being kept in an ice bath. The excess of alkali was sufficient to prevent the racemization of the substance. After all of the anhydride had been added, the solution was allowed to stand at room temperature for 15 minutes, and then 2.0 cc. of concentrated HCl were added. The precipitate was removed by filtration, washed with acidulated water, then recrystallized twice from dilute ethanol, and dried *in vacuo* over H_2SO_4. The yield was 760 mg., or about 67 per cent of the theoretical amount.

	C	H	Acetyl	M.p.
Found	43.25	4.32	12.80	118–119
$BrC_{12}H_{15}O_3NS$. Calculated .	43.24	4.54	12.91	

1.0 per cent of the substance in 95 per cent ethanol had a specific rotation of $[\alpha]_D^{24} = -37°$.

N-Acetyl-S-*p*-bromobenzyl-*dl*-cysteine was similarly prepared, with excess acetic anhydride. The racemic compound melted at 151–152°, and 2 per cent of the substance in N NaOH had no optical activity.

S-p-Bromobenzylglutathione—Because of the high cost of commercial glutathione, S-*p*-bromobenzylglutathione was prepared by direct benzylation of an aqueous extract of bakers' yeast. The

preparation of S-benzylglutathione was described by us previously (15). 1 pound of fresh bakers' yeast was thoroughly mixed for 10 to 15 minutes with 1 liter of acetone and the mixture was filtered. The yeast was then thoroughly mixed for 30 minutes with 150 cc. of water and the mixture was filtered with the aid of Filter Cel. The extraction was repeated with another 150 cc. of water for 15 minutes, and the extracts were combined. The latter were made alkaline to litmus with 2.5 N NaOH, and an excess of 5 cc. of the alkali was added, followed by 2 cc. of 0.8 per cent alcoholic solution of p-bromobenzyl bromide. The addition of the bromide and alkali, with shaking, was continued until a total of 10 cc. of the bromide solution and 10 cc. of the alkali had been added in about 15 minutes. The mixture was extracted with ether, and the aqueous layer was acidified with HCl to pH 6.0. After standing in the refrigerator overnight, the granular material was removed by filtration, washed with cold water, then recrystallized twice from boiling water, and dried *in vacuo* over sulfuric acid. The yield was 240 mg.

	C	H	N	S	M.p.
Found	42.91	4.78	8.91	6.66	199–201
$BrC_{17}H_{23}O_6N_3S$. Calculated.... ..	42.79	4.82	8.81	6.71	

This procedure offers the possibility of preparing glutathione from natural sources, avoiding the use of H_2S and Cu_2O. Glutathione can be obtained from the benzyl derivative by debenzylation in liquid ammonia with sodium (16).

Feeding Experiments with Rats

A different group of rats was used in each experiment described below. The rats were born and raised in this laboratory and had not been experimented upon previously.

p-Bromobenzyl Bromide—Six male adult rats of 250 gm. average weight were kept in individual metabolism cages and fed a complete synthetic casein diet which contained 0.1 per cent of p-bromobenzyl bromide. The rats ate the food reluctantly (about 5.0 gm. of food per day per rat).[2] The urine was collected each 3rd day over a period of 9 days, and kept in the refrigerator.

[2] Four rats of the ten selected refused to eat the food which contained p-bromobenzyl bromide.

Upon acidification of the urine with a few drops of concentrated HCl (strongly acid to Congo red paper), a white precipitate formed. It was collected, washed with cold water, and then recrystallized twice from boiling water and finally from dilute ethanol. The substance had the appearance of short needles. The yield from 240 mg. of p-bromobenzyl bromide consumed by six rats was 73 mg.

	C	H	N	Acetyl	M.p.
Found .	43.32	4.38	4.14	12.86	118–119
$BrC_{12}H_{15}O_3NS$. Calculated ...	43.24	4.54	4.20	12.91	118–119

1.0 per cent of the isolated mercapturic acid in 95 per cent ethanol had a specific rotation of $[\alpha]_D^{24} = -36.7°$. A mixture of the substance with the synthetic N-acetyl-S-p-bromobenzyl-l-cysteine showed no depression of the melting point.

S-p-Bromobenzyl-l-Cysteine—200 mg. of the substance mixed with 50 gm. of the casein diet were fed over a period of 4 days to each of the six rats. N-Acetyl-S-p-bromobenzyl-l-cysteine was isolated from the urine as was described above. The yield was about 48 per cent of the amount of S-p-bromobenzylcysteine fed.

	C	H	Acetyl	M.p.	$[\alpha]_D^{24}$
Found ..	43.40	4.40	12.77	118–119	−37.3

S-p-Bromobenzylglutathione—50 mg. of p-bromobenzylmercapturic acid were isolated from the urine of three rats which, in a 2 day period, ingested 200 mg. of the glutathione derivative mixed with food. The procedure for the isolation and purification of the substance was the same as that described above.

	C	H	Acetyl	M.p.	$[\alpha]_D^{24}$
Found . . .	43.20	4.46	12.69	118–119	−37.0

DISCUSSION AND SUMMARY

The present work demonstrates the synthesis of p-bromobenzyl-mercapturic acid in the rat from p-bromobenzyl bromide, S-p-bromobenzyl-l-cysteine, and S-p-bromobenzylglutathione. The same considerations and deductions which we made previously regarding the possible origin of cysteine of the mercapturic acid (11, 13), the mechanism of acetylation of the cysteine derivative (14), and mercapturic acid formation from benzylated glutathione

(15) are applicable to the new mercapturic acid. Preliminary work also indicates that p-bromobenzyl bromide inhibits the growth of young rats which are maintained on a low casein diet. Administration of cystine, methionine, or glutathione, together with the bromide, promptly alleviates the inhibition of growth (unpublished data).

The author wishes to express his thanks to Mr. J. Alicino for the microanalytical work.

BIBLIOGRAPHY

1. Abderhalden, E , and Wertheimer, E., *Arch. ges. Physiol.*, **207**, 215 (1925).
2. Baumann, E., and Preusse, C., *Ber. chem. Ges.*, **12**, 806 (1879).
3. Bourne, M. C., and Young, L., *Biochem. J.*, **28**, 803 (1934).
4. Boyland, E., and Levi, A., *Biochem. J.*, **30**, 1225 (1936).
5. Jaffe, M., *Ber. chem. Ges.*, **12**, 1092 (1879).
6. Lawrie, N. R , *Biochem J.*, **25**, 1037 (1931).
7. Nishimura, K., *Acta schol. med. univ. imp. Kioto*, **12**, 73 (1929–30).
8 Stekol, J. A., *J. Biol. Chem.*, **110**, 463 (1935).
9. Stekol, J. A., *Proc. Soc. Exp. Biol. and Med.*, **33**, 115 (1935).
10. Stekol, J. A., *J. Biol. Chem.*, **113**, 675 (1936).
11. Stekol, J. A., *J. Biol. Chem.*, **117**, 147 (1937); **118**, 155 (1937).
12. Stekol, J. A., *J. Biol Chem.*, **121**, 87 (1937).
13. Stekol, J. A., *J. Biol. Chem ,* **121**, 93 (1937).
14. Stekol, J. A., *J. Biol. Chem.*, **124**, 129 (1938); **128**, 199 (1939).
15. Stekol, J. A., *Proc. Soc. Exp. Biol. and Med.*, **43**, 108 (1940).
16. du Vigneaud, V., and Miller, G. L., *J. Biol. Chem.*, **116**, 469 (1936).
17. Virtue, R. W., *Proc. Soc. Exp Biol and Med.*, **34**, 425 (1936).
18. White, A., and Jackson, R W., *J. Biol. Chem.*, **111**, 507 (1935).

KINETICS OF PROTEINASE ACTION

APPLICATION TO SPECIFICITY PROBLEMS

By GEORGE W. IRVING, Jr., JOSEPH S. FRUTON, AND
MAX BERGMANN

(*From the Laboratories of The Rockefeller Institute for Medical Research,
New York*)

Heretofore, proteins have been the only substrates used for the quantitative study of the reaction kinetics of proteinase action (1). In the enzymatic hydrolysis of a protein, numerous peptide linkages are split. These peptide linkages differ from one another in the structure of the amino acids involved as well as in other structural details of the adjacent sections of the peptide chain. It has been observed that such structural differences greatly influence the rate at which proteinases hydrolyze peptide linkages (2). Consequently, in the hydrolysis of a protein molecule by a proteinase it cannot be expected that the various peptide bonds are hydrolyzed at identical rates. The experimentally measured rate of hydrolysis must therefore represent the resultant of the unlike rates of numerous simultaneous and consecutive individual reactions.

In recent years there have been synthesized a number of substrates of known structure that are hydrolyzed by proteinases with the scission of only one peptide linkage per substrate molecule. Several of these substrates have been employed in the experiments reported in this paper to obtain the unambiguous kinetics of the splitting of a single peptide bond by a proteinase. In a reaction of this kind it is to be expected that, at constant temperature and constant pH, first order kinetics will be found if no appreciable inactivation of the enzyme occurs throughout the measured course of the reaction and if the products of the reaction do not inhibit the enzymatic action. In the case of intracellular proteinases that require activation, an amount of activator sufficient to insure maximal activation of the enzyme must also be employed. Experiments performed under these conditions have shown that first order kinetics are actually obtained for the hy-

drolysis of simple substrates by crystalline trypsin and the intra-
cellular enzymes papain, ficin, and beef spleen cathepsin.

<div align="center">Table I</div>

Kinetics of Hydrolysis of Benzoylarginineamide* by Cysteine-Papain

The papain preparation employed was Papain B (*cf.* the experimental
section).

Enzyme con-centration	Time	Hydrolysis	K (first order)†	Average K	c_{BAA}
mg. protein N per cc.	*min.*	*per cent*			
0.0025	45	19	0.0020		
	60	22	0.0018		
	75	28	0.0019		
	90	37	0.0022		
	105	39	0.0020		
	120	44	0.0021	0 0020	0.80
0.005	15	12	0.0037		
	30	19	0.0036		
	45	27	0.0031		
	60	36	0.0033		
	75	47	0.0037		
	90	53	0.0037		
	105	58	0.0036		
	120	62	0.0035	0.0035	0.70
0.01	15	21	0.0069		
	30	40	0.0074		
	45	53	0.0073		
	60	67	0.0080		
	75	73	0.0076		
	90	81	0.0080	0.0075	0.75
0.02	15	39	0.0144		
	30	68	0.0165		
	45	77	0.0142	0.0150	0.75
Average.............					0.75

* This substrate was synthesized as described in a previous paper (4).
† $K = 1/t \log a/(a - x)$.

The kinetics of crystalline trypsin are described in another
communication (3); data on the hydrolysis of benzoyl-*l*-arginine-
amide by the cysteine-activated enzymes papain, ficin, and beef
spleen cathepsin are reported in Tables I, II, and III.   These

data show that in each case first order velocity constants are obtained and, further, that these constants are proportional to the enzyme concentrations. The results reported in Table IV demonstrate that an additional prerequisite of a first order reaction is fulfilled; namely, that the velocity constant is independent of the

TABLE II

Kinetics of Hydrolysis of Benzoylarginineamide by Cysteine-Ficin

Enzyme concentration	Time	Hydrolysis	K (first order)	Average K	c_{BAA}
mg. protein N per cc.	*min.*	*per cent*			
0 0075	60	27	0.0023		
	120	47	0.0023		
	240	71	0.0022		
	300	78	0 0022		
	360	85	0.0023	0.0023	0.30
0.015	30	27	0.0046		
	60	44	0.0042		
	90	58	0 0042		
	120	71	0.0045		
	240	92	0.0046	0.0044	0.29
0.0225	30	39	0.0072		
	60	65	0.0076		
	90	77	0.0071		
	120	84	0.0066		
	150	91	0.0070	0.0071	0.31
0.03	30	46	0.0089		
	60	72	0.0092		
	90	85	0.0092		
	120	91	0.0087		
	150	94	0.0082	0.0088	0.29
Average......			....		0.30

initial concentration of the substrate within the limits of the substrate concentrations employed.

Kinetics of a first order reaction have also been found for the hydrolysis of hippurylamide and carbobenzoxy-*l*-leucylglycylglycine by cysteine-papain (Table V). However, when carbobenzoxy-*l*-isoglutamine is used as the substrate (Table VI), first order constants are obtained with cysteine-papain only up to about 60 per cent hydrolysis. Above 60 per cent hydrolysis the rate de-

clines sharply.   The constants calculated from the splittings below 60 per cent are proportional to the enzyme concentration.

The data presented in Tables I to VI show that, despite the doubtful homogeneity of the available intracellular enzymes, it

TABLE III

Kinetics of Hydrolysis of Benzoylarginineamide by Cysteine-Beef Spleen "Cathepsin"

Enzyme concentration	Time	Hydrolysis	K (first order)	Average K	C_{BAA}
mg. protein N per cc.	min.	per cent			
0.11	60	12	0.00093		
	120	21	0.00086		
	180	33	0.00096		
	240	39	0.00091		
	300	46	0.00089	0.00091	0.0082
0.22	60	22	0.0018		
	120	38	0.0017		
	180	50	0.0017		
	240	61	0.0017		
	300	68	0.0016	0.0017	0.0078
0.44	30	21	0 0034		
	60	35	0.0031		
	90	47	0.0031		
	120	60	0.0033		
	180	73	0 0032	0.0032	0.0074
0.66	30	29	0 0050		
	60	48	0.0048		
	90	62	0.0047		
	120	73	0.0047		
	180	83	0.0043	0.0047	0.0072
0.88	30	36	0.0065		
	60	58	0.0063		
	90	73	0.0063		
	120	82	0.0062	0.0063	0.0072
Average.	..	. ...	. . ..	. .....	0.0076

is possible to select synthetic substrates that will be hydrolyzed with first order kinetics.

Proteolytic Coefficient—If, as in the above cases, the rate constants K_1, K_2, K_3,...for a given enzyme preparation and a given

substrate are proportional to the enzyme concentrations E_1, E_2, E_3,...where E_1, E_2, E_3,...are expressed in mg. of protein N per

TABLE IV

Kinetics and Concentration of Benzoylarginineamide

Enzyme	Enzyme concentration	Substrate concentration	K (first order)
	mg. protein N per cc.	*mM per cc.*	
Cysteine-Papain A*	0.01	0.02	0.0013
		0.035	0.0012
		0.05	0.0011
		0.0625	0.0011
		0.075	0.0011
Cysteine-ficin†	0.01	0.02	0.0025
		0.05	0.0025
		0.075	0.0023

* Papain A was obtained by precipitation with isopropyl alcohol (*cf.* the experimental section).

† An interval of 4 weeks elapsed between these experiments and those reported in Table II.

TABLE V

Kinetics of Hydrolysis of Simple Substrates by Cysteine-Papain

The papain preparation employed was Papain A (*cf.* the experimental section).

Substrate	Enzyme concentration	Time	Hydrolysis	K (first order)
	mg. protein N per cc.	*min.*	*per cent*	
Hippurylamide	0.04	120	20	0.00080
		180	31	0.00089
		240	37	0.00084
		360	47	0.00077
		420	54	0.00080
Carbobenzoxy-*l*-leucylglycylglycine (*cf.* (5))	0.05	65	25	0.0019
		90	32	0.0019
	0.10	60	41	0.0038
		90	54	0.0037
	0.15	60	57	0.0061
		90	69	0.0057

cc. of test solution, then the quotients K_1/E_1, K_2/E_2, K_3/E_3... have the same numerical value. This value represents the rate

constant for the action of the enzyme preparation upon the substrate S, when the enzyme preparation is present in a concentration of 1 mg. of protein N per cc. of test solution. The numerical value of K/E may be designated as the proteolytic coefficient C_S of the enzyme preparation, with respect to its action upon the

TABLE VI

Kinetics of Hydrolysis of Carbobenzoxyisoglutamine* by Cysteine-Papain

The papain preparation employed was Papain A (*cf.* the experimental section).

Enzyme concentration	Time	Hydrolysis	K (first order)	Average K	C_{CIG}
mg. protein N per cc.	*min.*	*per cent*			.
0.04	60	18	0.0014		
	120	31	0.0013		
	196	42	0.0012		
	240	50	0.0013		
	305	57	0.0012	0.0013	0.033
0.06	60	25	0.0021		
	120	40	0.0019		
	193	53	0.0017		
	240	61	0.0017	0.0019	0.032
	360	65	(0.0013)		
0.08	60	31	0.0027		
	92	47	0.0030		
	120	50	0.0025		
	180	66	0.0026	0.0027	0.034
	360	77	(0.0018)		
0.10	60	39	0.0036		
	90	50	0.0034		
	120	57	0.0031	0.0034	0.034
	180	65	(0.0025)		
	360	82	(0.0021)		

The figures in parentheses are not included in the averages.

* This substrate was synthesized as described in an earlier paper (6).

particular substrate S. The numerical value C_S of a given enzyme preparation must obviously depend upon the amount of other proteins present. For example, a calculation from the data on the kinetics of the hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine by a carboxypeptidase preparation recrystallized four times (7) shows that the enzyme had a proteolytic coefficient,

$C_{\text{CGP}} = 13.2$.   If an enzyme can be purified to such a degree that all other proteins have been removed, then C_S will assume a maximum value that may be designated as the specific proteolytic coefficient $[C]_S$ of the enzyme with respect to its action upon the substrate S.   For the few proteolytic enzymes that are available at present in a pure state, the specific proteolytic coefficients toward a number of substrates may be determined with a considerable degree of accuracy.   The values thus obtained are a quantitative representation of the specificity of the enzyme in question.[1]

When the specific proteolytic coefficient of a given enzyme is known, the percentage of active enzyme contained in a partly purified preparation may be ascertained by means of the quotient $(100 \times C_S)/[C]_S$.

At the present time the majority of proteolytic enzymes cannot be obtained in such a degree of purity that their specific proteolytic coefficients may be determined.   However, the proteolytic coefficients of such impure enzyme preparations may be used as valuable tools for the quantitative study of the enzymatic components of these impure preparations.

Proteolytic Quotient—It may be assumed that a given enzyme preparation P splits a substrate $S1$ with a proteolytic coefficient C_{S1} and another substrate $S2$ with a proteolytic coefficient C_{S2}. The proteolytic quotient $Q = C_{S1}/C_{S2}$ expresses the fact that $S1$ is hydrolyzed Q times faster than $S2$ by the above enzyme preparation.   The quotient Q may be utilized to determine whether the substrates $S1$ and $S2$ are split by the same or different enzymatic components of the above preparation P.   If P contains only a single proteolytic enzyme, this enzyme must be responsible for the hydrolysis of the two substrates.   Then Q should not change its numerical value when P is subjected to fractionation procedures. If, however, more than one enzymatic component of P participates in the hydrolysis of the two substrates, then the relative amounts of these components might be changed by fractionation procedures and consequently Q should change its numerical value.   Conversely, if as a result of the fractionation of an enzyme preparation the proteolytic quotient changes markedly, then the splitting of the two substrates involved may be attributed to more than one

[1] The above value of the proteolytic coefficient C_{CGP} of carboxypeptidase may also represent the specific proteolytic coefficient of this enzyme.

component of the enzyme preparation. A similar conclusion would apply when two enzyme preparations of different origin are compared. In a subsequent paper (8) this approach has been employed for the study of the composite nature of beef spleen cathepsin.

Quantitative Determination of Intracellular Enzymes. Units of Enzymatic Activity

It has already been pointed out that the measured rate of hydrolysis of a protein by a proteinase represents the resultant of the unlike rates of numerous simultaneous and consecutive reactions. Complicated reaction rates of this type do not permit a decision as to whether an enzyme preparation hydrolyzes the protein through the action of a single proteinase or several proteinases.[2] Moreover, the use of proteins as substrates does not allow one to differentiate and to determine separately the single proteinase components contained in enzyme mixtures as obtained from natural sources. The use of the simple substrates for proteinases is not accompanied by these disadvantages. Therefore, whenever first order constants are obtained with synthetic substrates and these constants are proportional to the enzyme concentration, it is possible to employ such simple substrates for the characterization and separate estimation of each proteinase component of enzyme mixtures obtained from animal or plant tissues. In these cases, the reaction rate constants of the hydrolysis of the simple substrates may be used for the definition of units of proteinase activity. For example, the enzymatic unit for the component of cysteine-papain that acts on benzoyl-l-arginine-amide may be defined as the quantity of enzyme that splits the substrate, under the conditions described in the experimental

---

[2] Several methods have been designed to measure, by means of protein precipitants, the rate of enzymatic transformation of protein material into non-protein material. While some of these methods have proved to be of empirical value, they offer no indication whether this transformation is performed by a single proteinase or several proteinases. Furthermore, the assumption that the increase in non-protein N is proportional to the number of peptide bonds hydrolyzed appears to have no sound basis. The amount of non-protein N liberated is determined by the position of the hydrolyzed peptide bond within the protein molecule and also by the nature of the split-products.

section, with a first order constant of 0.0020. The same value of K may be taken to define the units of other components of papain as well as the other intracellular proteinases. Thus, 1 unit of cysteine-papain (benzoyl-l-arginineamide) gives K (first order) = 0.0020, 1 unit of cysteine-ficin (benzoyl-l-arginineamide) gives K (first order) = 0.0020, and 1 unit of cysteine-beef spleen cathepsin (benzoyl-l-arginineamide) gives K (first order) = 0.0020. The enzyme content per mg. of protein N of the enzyme preparation for the proteinases studied in Tables I, II, and III is therefore cysteine-papain (benzoyl-l-arginineamide), 375 units per mg. of protein N, cysteine-ficin (benzoyl-l-arginineamide), 150 units per mg. of protein N, and cysteine-beef spleen cathepsin (benzoyl-l-arginineamide), 3.8 units per mg. of protein N.

Anomalous Relationship between Rate Constant and Enzyme Concentration

In the experiments described earlier in this paper the reaction constants were found to be proportional to the enzyme concentration. Further experiments with synthetic substrates led to the observation that this simple relationship does not always hold. Where such deviations appear, they show that the proportionality relationship is complicated by additional factors that merit closer investigation.

It is well known that even highly purified preparations of the intracellular enzymes show some proteolytic activity in the absence of added activators. Experiments with unactivated ficin have shown that the hydrolysis of benzoyl-l-arginineamide follows first order kinetics for each enzyme concentration; however, as the enzyme concentration is increased, the corresponding increase in the value of K is greater than should be expected if a true proportionality exists between the rate constant and the enzyme concentration (Table VII).

Experiments in which HCN has been employed as the activator for ficin and papain also yield satisfactory rate constants for each enzyme concentration but these constants again increased faster than did the enzyme concentration. On the other hand, when thioglycolic acid is used as the activator, the first order constants obtained are proportional to the enzyme concentration, as in the case of cysteine activation.

The fact that cysteine-papain (benzoyl-l-arginineamide) obeys the proportionality relationship, while HCN-papain (benzoyl-l-arginineamide) does not, reveals quantitative differences in the action of the two most widely used activators for papainases. It follows, therefore, that quantitative studies on proteolytic enzymes and activator-enzyme combinations should always be preceded by an investigation of the nature of the rate constant-enzyme concentration relationship.

TABLE VII

Kinetics of Hydrolysis of Benzoylarginineamide by Ficin, HCN-Ficin, HCN-Papain, and Thioglycolic Acid-Ficin

Enzyme	Activator	Enzyme concentration	K (first order)	c_{BAA}
		mg. protein N per cc.		
Ficin	None	0.02	0.00015	0.0075
		0.04	0.0024	0.06
		0.06	0.0050	0.083
		0.12	0.0191	0.16
	HCN	0.02	0.0020	0.10
		0.03	0.0044	0.15
		0.04	0.0081	0.20
		0.06	0.0130	0.22
Papain C	"	0.0025	0.0009	0.36
		0.005	0.0019	0.38
		0.01	0.0054	0.54
Ficin	Thioglycolic acid	0.02	0.0038	0.19
		0.03	0.0051	0.17
		0.04	0.0066	0.17

EXPERIMENTAL

Papain A—150 gm. of a good grade of Ceylon papain were finely ground and shaken for 1 hour with 3 liters of distilled water. The filtrate (2780 cc.) was made 0.4 saturated with ammonium sulfate by the addition of 673 gm. of the salt. After 2 hours stirring in the cold room, 3 gm. of Filter Cel were added and the solution was filtered. The filtrate (3030 cc.) was treated with 621 gm. of ammonium sulfate and the stirred solution was allowed to stand overnight in the cold. The precipitate, collected by centrifugation, was dissolved in 1 liter of distilled water and 410 gm. of

ammonium sulfate were added. After 15 hours in the cold, a small amount of Filter Cel was added and the precipitate was filtered off by suction. The precipitate was dissolved in 400 cc. of water. The Filter Cel was removed by filtration and washed thoroughly with water. The filtrate and washings (680 cc.) were cooled and treated, portionwise, with 2040 cc. of cold isopropyl alcohol. The precipitate was allowed to settle in the cold overnight. It was filtered off by suction and washed with four 100 cc. portions of cold isopropyl alcohol followed by four 100 cc. portions of ether. The washed product (Papain A) was dried in the cold in a vacuum desiccator over calcium chloride. Yield, 42.5 gm.

Papain B—180 gm. of crude papain were fractionated by the procedure outlined by Balls and Lineweaver (9) for the preparation of crystalline papain from papaya latex. No cyanide was used. The final product (670 mg.) showed, in suspension, a crystalline sheen, although no crystals could be discerned under the microscope. Balls and Lineweaver obtained their crystalline papain from fresh latex.

Ficin—We are indebted to Dr. R. T. Major and Dr. A. Walti for the sample of crystalline ficin (10).

Cathepsin (Beef Spleen)—This material was prepared as described in a previous communication (11).

Procedure

Hydrolyses were carried out in 2.5 cc. volumetric flasks at a bath temperature of 40°. The extent of hydrolysis was determined on successive 0.2 cc. samples by the titration method of Grassmann and Heyde (12). Except for the results given in Table IV, the substrate was present in a concentration of 0.05 mM per cc. The papain and ficin experiments were buffered at pH 5.0 by the addition of 0.1 cc. of 0.2 M citrate buffer per cc. In the cathepsin experiments 0.1 cc. of citrate buffer was used per cc. of test solution to maintain the pH at 4.5. In each case, preliminary experiments were conducted to determine the amount of activator necessary to produce maximal activation of the enzyme. The required amounts of each activator per cc. of test solution were as follows: cysteine 0.020 mM, thioglycolic acid 0.040 mM, HCN 0.020 mM. With HCN and thioglycolic acid it was found necessary to

employ a 2 hour incubation of enzyme and activator at 40° before the substrate was added, in order to obtain maximal splitting values.   With these two activators also, it was necessary to use separate flasks for each time interval measured, since the successive sampling of a single flask, as was done in the cysteine experiments, resulted in a progressive decrease in the reaction velocity.   Enzyme blanks were performed throughout.

BIBLIOGRAPHY

1. Oppenheimer, C., Die Fermente und ihre Wirkungen, The Hague, 5th edition, suppl., 794, 868, 887 (1936).
2. Bergmann, M., *Chem. Rev.*, **22,** 423 (1938).
3. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **138,** 243 (1941).
4. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127, 643** (1939).
5. Bergmann, M., Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, **111,** 225 (1935).
6. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65,** 1192 (1932).
7. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134,** 225 (1940).
8. Fruton, J. S., Irving, G. W., Jr , and Bergmann, M., *J. Biol. Chem.*, **138,** 249 (1941).
9. Balls, A. K., and Lineweaver, H., *J. Biol. Chem.*, **130,** 669 (1939).
10. Walti, A., *J. Am. Chem. Soc.*, **60,** 493 (1938).
11. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130,** 19 (1939).
12. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183,** 32 (1929).

THE KINETICS OF THE ACTION OF TRYPSIN UPON
SYNTHETIC SUBSTRATES

By KLAUS HOFMANN* AND MAX BERGMANN

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

Three substrates of low molecular weight are known to be hydrolyzed by crystalline beef trypsin: benzoyl-*l*-arginineamide (1), benzoylglycyl-*l*-lysineamide, and benzoyl-*l*-lysineamide (2). In a search for additional substrates that are hydrolyzed by this enzyme, benzoylglycyl-*l*-arginineamide was synthesized with nitroarginine (3) as the starting material. Benzoylglycyl-*l*-arginineamide was characterized by means of its crystalline picrate.

It has been found that the tryptic digestion of benzoylarginineamide, benzoylglycyllysineamide, and benzoylglycylarginineamide follows the kinetics of a first order reaction and that the reaction constants are proportional to the trypsin concentration. Two preparations of crystalline trypsin gave the same proteolytic coefficient (first order constant ÷ enzyme concentration) for the hydrolysis of benzoylarginineamide; namely, $C_{BAA} = 0.038$ (Table I). With benzoylglycyllysineamide as substrate, the proteolytic coefficient was found to be $C_{BGLA} = 0.22$ (Table II). With benzoylglycylarginineamide as substrate, the proteolytic coefficient was found to be $C_{BGAA} = 0.40$ (Table III). It may be regarded as probable that these proteolytic coefficients also represent the specific proteolytic coefficients (4) of pure beef trypsin. However, further experiments are desirable with regard to this question.

In accordance with the principles discussed in the preceding communication (4), the quotients $C_{BGLA}/C_{BAA} = 0.22/0.038 = 5.8$, $C_{BGAA}/C_{BGLA} = 0.40/0.22 = 1.8$, and $C_{BGAA}/C_{BAA} = 0.40/0.038 = 10.5$ must be regarded as characteristic properties describing the specificity of beef trypsin.

* Fellow of the Rockefeller Foundation

TABLE I

Hydrolysis of Benzoyl-l-Arginineamide by Trypsin

Enzyme	Time	Hydrolysis at pH 7.5 and 25°	$K = \frac{1}{t} \log \frac{a}{a-x}$	c_{BAA}
mg. N per cc.	*min.*	*per cent*		
0.015*	60	8	0.00060	
	120	16	0.00063	
	180	23	0.00063	0.041
	240	29	0.00062	
0.020†	60	9	0.00068	
	120	17	0.00068	
	180	24	0.00066	0.034
	240	31	0.00067	
	360	44	0.00070	
0.030*	60	14	0.0011	
	120	26	0.0011	
	180	37	0.0011	0.037
	240	46	0.0011	
0.040†	60	18	0.0014	
	120	34	0.0015	
	180	46	0.0015	0.036
	240	55	0.0014	
0.045*	60	22	0.0018	
	120	38	0 0017	
	180	51	0.0017	0 039
	240	62	0.0018	
0.060†	60	25	0.0021	
	120	45	0.0022	
	180	60	0.0022	0.037
	240	71	0.0022	
0.060*	60	28	0.0024	
	120	48	0.0024	
	180	63	0.0024	0.040
	240	74	0.0024	
0.080†	60	33	0.0029	
	90	46	0.0030	
	120	55	0.0029	
	150	62	0.0028	0.036
	180	72	0.0031	
	210	74	0.0028	
0.100†	60	41	0.0038	
	90	54	0.0037	
	120	64	0.0037	0.038
	150	71	0.0036	
	180	82	0.0041	
Average				0.038

* Crystalline trypsin, dried at 5° and instantly used.
† Crystalline trypsin, stored 1 year at 5°.

The enzymatic unit for beef trypsin may be defined as the quantity of enzyme that will split benzoyl-*l*-arginineamide, under the conditions described in Table I, with a first order constant of 0.0020. On this basis, the enzyme content of the trypsin preparations studied in Table I is 19 units per mg. of protein N.

TABLE II

Hydrolysis of Benzoylglycyl-l-Lysineamide by Crystalline Trypsin

Enzyme*	Time	Hydrolysis at pH 7 5 and 25°	$K = \frac{1}{t} \log \frac{a}{a-x}$	c_{BGLA}
mg. N per cc.	min.	per cent		
0.0037	120	18	0.00072	
	180	29	0.00083	0.21
	240	36	0.00081	
0.0074	60	21	0.0017	
	90	29	0.0017	
	120	38	0.0017	
	150	44	0.0017	0.23
	180	51	0.0017	
	240	61	0.0017	
0.0111	30	17	0.0027	
	60	32	0.0028	
	90	43	0.0027	
	120	51	0.0026	0.23
	180	65	0.0025	
	210	71	0.0026	
	240	73	0.0024	
0.0148	30	22	0.0036	
	60	38	0.0035	
	90	52	0.0035	
	120	60	0.0033	0.22
	150	66	0.0031	
	180	71	0.0030	
	240	77	0.0027	
Average	....			0.22

* Crystalline trypsin, stored 1 year at 5°.

Finally, it may be mentioned that benzoylglycylarginineamide is hydrolyzed by cysteine-papain into benzoylglycine and arginineamide.

The authors wish to express their thanks to Mr. Stephen M. Nagy for the analyses reported in this paper.

Benzoylglycyl-(Nitro-l-Arginine)—To an ice-cold solution of 2.2 gm. of nitro-*l*-arginine (3) in 10 cc. of N NaOH, 2 gm. of hippuryl chloride and 14 cc. of N NaOH were added in small portions with vigorous stirring.   The reaction mixture was filtered and acidified

TABLE III

Hydrolysis of Benzoylglycyl-l-Arginineamide by Crystalline Trypsin and by Cysteine-Papain

Enzyme	pH	Temperature	Time	Hydrolysis	$K = \dfrac{1}{t} \log \dfrac{a}{a-x}$	C_{BGAA}	Isolation of products
mg. N per cc.		°C.	min.	per cent			
0.0037*	7.5	25	60	19	0.0015		
			120	32	0.0014	0.42	
			180	46	0.0015		
			240	62	0.0018		
0.0074*	7.5	25	60	33	0.0029		
			120	54	0.0028	0.38	
			180	69	0.0028		
			240	79	0.0028		
0.0111*	7 5	25	30	27	0.0046		
			60	47	0.0046	0.41	
			120	71	0.0045		
			180	84	0.0044		
			1200	100			NH₃, 90% of theory
0.040†	4.9	40	60	14	0.0011		
			120	25	0.0010		
			180	36	0.0011		
			240	44	0 0011		
			1200	98			Hippuric acid, 94% of theory

* Twice crystallized trypsin.
† Cysteine-papain prepared from dried latex.

with dilute HCl.   The resulting oil soon crystallized.   The crystals were filtered off, washed with water, and were recrystallized from 5 N acetic acid.   Long needles, which contained 1 molecule of water of crystallization, were obtained.   Yield, 2.2 gm.

$C_{15}H_{20}O_6N_6 \cdot H_2O$.   Calculated.   C 45.2, H 5.6, N 21.1, H₂O 4.5
398.2   Found.   " 45.3, " 5.5, " 21.2, "   4.5

The monohydrate sintered at 145° and melted at 158–161° with decomposition. At 100° *in vacuo* over P_2O_5 the compound was obtained in an anhydrous and rather hygroscopic form, which melted at 140–142°.

Benzoylglycyl-(Nitro-l-Arginine)-Amide—15 gm. of hippuryl-*l*-nitroarginine were dissolved in 120 cc. of methanol and saturated with dry HCl gas at 0°. The solution was kept in the ice box overnight. The methanol was then removed *in vacuo*, the resulting oil was redissolved in methanol, and this solution was again treated with HCl. After removal of the solvent *in vacuo*, the hippuryl-*l*-nitroarginine methyl ester was obtained as an oil. It was transformed into the corresponding amide in the usual manner. The amide was purified by recrystallization from 5 N acetic acid. Clusters of needles of the monohydrate were obtained, which melted at 144–150°. Yield, 10 gm.

$C_{15}H_{21}O_5N_7 \cdot H_2O$. Calculated. C 45.3, H 5.8, N 24.7, H_2O 4.5
397.2   Found.   " 45.3, " 5.7, " 24.7, " 4.5

The monohydrate, on drying at 100° *in vacuo* over P_2O_5, yielded the anhydrous compound, m.p. 148–152°.

Benzoylglycyl-l-Arginineamide—1 gm. of hippuryl-*l*-nitroarginineamide was suspended in a mixture of 50 cc. of 5 N acetic acid and 5.5 cc. of N HCl, and was hydrogenated in the presence of palladium black. After 4 moles of hydrogen had been absorbed, the catalyst was filtered off and the solution was evaporated to dryness *in vacuo*. To remove the excess of acetic acid, 20 cc. of water were added and the solution was concentrated to dryness *in vacuo*. This procedure was repeated three times. The residue was taken up in 20 cc. of water, and the solution was made alkaline to phenolphthalein with aqueous $Ba(OH)_2$ and was concentrated *in vacuo* to one-third of its volume. The barium was removed with sulfuric acid and an excess of a saturated aqueous solution of picric acid was added to the filtrate. The picrate was recrystallized from water. Long, orange needles, which melted between 150–160° with decomposition, were obtained. Yield, 1.1 gm.

$C_{21}H_{25}O_{10}N_9 \cdot \frac{1}{2}H_2O$. Calculated. C 44.1, H 4 6, N 22.2, H_2O 1.6
572.4   Found.   " 44.1, " 4.6, " 22.2, " 1.7

The anhydrous compound was obtained at 100° *in vacuo* over P_2O_5. M.p., 166–168°.

To obtain the hydrochloride, 229 mg. of the picrate were dissolved in 40 cc. of 0.1 N HCl and the picric acid was removed by adsorption on wool according to the directions of Müller (5). The colorless solution was concentrated to dryness *in vacuo*, twice dissolved in 10 cc. of water, and again evaporated. Since the hydrochloride could not be obtained in crystalline form, it was dissolved in 30 cc. of water and this solution employed for the enzyme experiments.

Hydrolysis of Benzoylglycyl-l-Arginineamide by Cysteine-Papain —92.6 mg. of the hydrochloride were hydrolyzed at 40° for 24 hours by a cysteine-papain solution containing 0.04 mg. of protein N per cc. The hydrolysate was concentrated *in vacuo* and 42 mg. of hippuric acid (94 per cent of the theory) were isolated. The material was crystallized from hot water. M.p., 187–189°. The mixed melting point with hippuric acid was 188–190°.

$$C_9H_9O_3N \ (179.2). \quad \text{Calculated, N } 7.8; \text{ found, N } 7.8$$

Enzymatic Studies

The crystalline beef trypsin was prepared and recrystallized according to the directions of Kunitz and Northrop (6). The substrate concentration was 0.05 mM per cc. in all cases. In the trypsin experiments the solutions were buffered by means of M/30 phosphate buffer. In the papain experiments 0.2 M citrate buffer was used. All the pH values reported in this paper were measured by the glass electrode. The liberated carboxyl groups were measured by the method of Grassmann and Heyde (7).

BIBLIOGRAPHY

1. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939).
2. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 81 (1939).
3. Bergmann, M., Zervas, L., and Rinke, H., *Z. physiol. Chem.*, **224**, 40 (1934).
4. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).
5. Müller, H., *Z. physiol. Chem.*, **209**, 207 (1932).
6. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936).
7. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).

ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

II. THE COMPOSITE NATURE OF BEEF SPLEEN CATHEPSIN

By JOSEPH S. FRUTON, GEORGE W. IRVING, Jr., and
MAX BERGMANN

(*From the Laboratories of The Rockefeller Institute for Medical Research,
New York*)

The essential rôle of the proteolytic enzymes of animal tissues in the intracellular synthesis and transformation of proteins has been discussed in recent publications from this laboratory (1, 2) and from other laboratories (3, 4). Willstätter and Bamann introduced the term "cathepsin" to designate the tissue proteinase acting at weak acidities (5). They suggested that the cathepsins of various animal tissues might be identical. From the work of Waldschmidt-Leitz and coworkers (6) and Grassmann *et al.* (7) it has been generally concluded that cathepsin can be activated by sulfhydryl compounds. This point of view is disputed by Anson (4) who regards cathepsin as the only proteinase known to be present in animal cells and assumes that it is not activated by cysteine. Anson believes that the proteinase is accompanied by a peptidase part that is activated by cysteine.

In a previous publication from this laboratory, simple synthetic substrates were applied to the characterization of the proteolytic components of partially purified extracts of beef spleen (2). The investigation indicated the presence in spleen "cathepsin" of at least three types of proteolytic activity. The first type effected the hydrolysis of substrates that contain tyrosine or phenylalanine and glutamic acid—such as carbobenzoxy-*l*-glutamyl-*l*-tyrosine. This type of splitting occurred in the absence of cysteine and similar activators, but was, in some cases, accelerated by the addition of cysteine. The second type of proteolytic activity required the presence of cysteine as activator and split a large variety of acylated peptides, amino acid amides, and their acyl

derivatives—such as *l*-leucineamide, hippurylamide, benzoyl-*l*-arginineamide—and peptides. Finally, there was a third type of activity which effected the hydrolysis of amino acid amides and peptides in the presence of ascorbic acid.

The above differentiation of the three types of proteolytic activity in beef spleen extracts does not show whether each of the three types corresponds to a separate enzymatic individual. Consequently, to provide a sounder experimental basis for the characterization of the enzymatic components of beef spleen cathepsin,

TABLE I

Reaction Kinetics of Cathepsin I

Beef spleen cathepsin, 0.29 mg. of protein N per cc. of test solution; substrate, carbobenzoxy-*l*-glutamyl-*l*-tyrosine (8); pH 5.35.

Time	K (first order)* at 40°		K (first order) at 25°	
	No cysteine	Cysteine	No cysteine	Cysteine
min.				
30	0.0023	0.0025		
60	0.0020	0.0024	0.00092	0.00092
90		0.0022		
120	0.0014		0.00092	0.00100
150		0.0018		
210	0.0012			
240			0.00088	0.00088
300	0.0009		0.00086	0.00095
Average.........			0.00089	0.00094

* $K = 1/t \log a/(a - x)$.

the kinetics of the action of cathepsin on synthetic substrates were investigated.

Beef Spleen Cathepsin I—The typical substrate for this type of activity is carbobenzoxy-*l*-glutamyl-*l*-tyrosine which is split between the glutamyl and tyrosine residues. The kinetics of the hydrolysis of this compound by a beef spleen enzyme preparation were determined both in the presence and the absence of cysteine. The data in Table I show that when the hydrolysis is performed at 25°, satisfactory first order constants are obtained. At this temperature the numerical values for the rate constants are the same (within the precision of the method) in the presence and

absence of cysteine. When the hydrolysis is performed at 40°, the first order rate constants decrease during the course of the reaction; however, it will be noted that in the presence of cysteine the constants decrease less rapidly than in the absence of cysteine. These results indicate that Cathepsin I is easily inactivated by heat and that this inactivation is retarded by the presence of cysteine. In carrying out kinetic studies on Cathepsin I, it is therefore necessary to work at a temperature at which the inactivation is not appreciable. Furthermore, the fact that at 25° cysteine produces no acceleration in the rate of hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine indicates that Cathepsin I does not require the addition of cysteine for its maximal proteolytic activity.

TABLE II

Effect of Enzyme Concentration on Kinetics of Cathepsin I

Substrate, carbobenzoxy-*l*-glutamyl-*l*-tyrosine; pH 5.40; temperature 25°.

Enzyme concentration	K (first order)	$\dfrac{K}{\text{Enzyme concentration}}$
mg. protein N per cc.		
0.10	0.00029	0.0029
0.20	0.00059	0.0029₅
0.30	0.00089	0.0030
0.40	0.00116	0.0029
0.50	0.00144	0.0029

The data in Table II show that the first order constants for the splitting of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by Cathepsin I are proportional to the enzyme concentrations employed.

Beef Spleen Cathepsin II—It has already been shown that a component of beef spleen cathepsin, when activated by cysteine, hydrolyzes the substrate benzoyl-*l*-arginineamide at 40° with first order constants that are proportional to the enzyme concentration (9). In Table III data are presented for the hydrolysis of a different type of substrate, *l*-leucineamide, that is also hydrolyzed at pH 5 and also only in the presence of an added activator such as cysteine. The first order constants found for the hydrolysis of *l*-leucineamide are proportional to the enzyme concentrations employed. It is thus possible to utilize reaction kinetics to deter-

mine whether or not the hydrolysis of benzoyl-l-arginineamide and l-leucineamide is performed by the same enzymatic component of beef spleen cathepsin.

The proteolytic quotient C_{BAA}/C_{LA}[1] of the beef spleen enzyme solution was found to be 0.82 (Table IV). When this solution was dialyzed against distilled water in a Kunitz dialyzer for 2 hours, a flocculent precipitate appeared. The quotient C_{BAA}/C_{LA} of the clear filtrate was found to be 2.6, showing that during the dialysis the enzymatic activity toward l-leucineamide disappeared much more rapidly than did the activity toward benzoyl-l-arginine-amide. Considerably more of the activity toward l-leucineamide

TABLE III

Kinetics of Hydrolysis of l-Leucineamide by Cysteine-Beef Spleen Cathepsin*
pH 5.2; temperature 40°.

Enzyme concentration	K (first order)	$\dfrac{K}{\text{Enzyme concentration}}$
mg. protein N per cc.		
0.11	0.0011	0.0100
0.22	0.0020	0.0091
0.44	0.0042	0.0095
0.66	0.0060	0.0091
0.88	0.0082	0.0094
Average	. . .. .	0.0094

* The preparation of this substrate has been described in a previous paper (10).

had disappeared after 24 hours of dialysis against distilled water, since the quotient C_{BAA}/C_{LA} of the clear enzyme solution had risen to 43.

These results show clearly that the cysteine-activated hydrolysis of benzoyl-l-arginineamide and l-leucineamide is performed by different proteolytic enzymes. The enzyme that hydrolyzes benzoyl-l-arginineamide in the presence of cysteine may be designated Cathepsin II. It will be recalled that a considerable number of synthetic substrates are split by beef spleen cathepsin in the presence of cysteine. Quantitative studies of reaction kinetics

[1] The symbols used in describing the kinetics of the catheptic enzymes have been defined in a preceding paper (9).

should show whether these hydrolyses are all due to the action of Cathepsin II or to some hitherto unidentified enzymes. The enzyme that hydrolyzes l-leucineamide in the presence of cysteine is discussed further in a succeeding paragraph.

Beef Spleen Cathepsin III—The hydrolysis of amino acid amides such as l-leucineamide and peptides such as l-leucylglycylglycine by beef spleen cathepsin is catalyzed not only by cysteine but also by ascorbic acid (2). However, the presence of ascorbic

TABLE IV

Differentiation of Cathepsins II and III

Solution A of beef spleen cathepsin was dialyzed against distilled water for 2 hours at 4° in a Kunitz dialyzer. The flocculent precipitate that appeared was filtered off and the clear filtrate, Solution B, was employed for hydrolytic experiments. Another portion of Solution A was dialyzed as above for 24 hours to give enzyme Solution C. Temperature of hydrolytic experiments, 40°.

Enzyme solution	Protein N per cc. test solution	Activator	Benzoyl-l-arginineamide*		l-Leucineamide†		$\dfrac{c_{BAA}(Cy)}{c_{LA}(Cy)}$	$\dfrac{c_{LA}(Cy)}{c_{LA}(Asc)}$
			K (first order)	c_{BAA}	K (first order)	c_{LA}		
	mg.							
A	0.34	Cysteine	0.0027	0.0080	0.0033	0.0097	0.82	
		Ascorbic acid			0.00065	0.0019		5.1
B	0.41	Cysteine	0.0034	0.0083	0.0013	0.0032	2.6	
		Ascorbic acid			0.00027	0.00066		4.9
C	0.26	Cysteine	0.0020	0.0077	0.00047	0.00018	43	

* pH 4.9.

† pH 5.2.

acid does not enable the enzyme preparation to split acylamino acid amides such as benzoyl-l-arginineamide or acyl peptides such as carbobenzoxy-l-leucylglycylglycine.

In the dialysis experiments described above, the fate of the component that hydrolyzes l-leucineamide in the presence of ascorbic acid was also followed by means of kinetics. This enzyme may be designated Cathepsin III. It was thus possible to compare the proteolytic coefficients for the ascorbic acid-activated and cysteine-activated hydrolysis of l-leucineamide. It was found

that before dialysis for 2 hours against distilled water the quotient $C_{LA}(Cy)/C_{LA}(Asc)$ was 5.1.   After dialysis the quotient was 4.9, indicating that the ascorbic acid-activatable enzyme component (Cathepsin III) disappeared at a rate which was parallel to that of the cysteine-activatable component.[2]

It appears probable therefore that the hydrolysis of l-leucineamide in the presence of cysteine is due to Cathepsin III.   Additional evidence to support this conclusion is provided by the data presented in Table V.   When a beef spleen solution was heated at 65° for 10 minutes, the enzymatic activity toward l-leucineamide in the presence of cysteine or ascorbic acid decreased markedly in comparison with that of the original enzyme solution. However, the values for the quotient $C_{LA}(Cy)/C_{LA}(Asc)$ did not change appreciably.

Further data in Table V indicate that l-leucylglycine is also hydrolyzed by Cathepsin III in the presence of cysteine or ascorbic acid.   On dialysis against distilled water for 2 hours the quotient $C_{LA}(Cy)/C_{LG}(Cy)$ was 1.7, whereas before dialysis the value was 2.0.   Furthermore, the quotient did not change appreciably after the enzyme solution was heated at 65°.   The question may also be raised whether Cathepsin III is responsible for the hydrolysis of peptides such as l-leucylglycylglycine in the presence of ascorbic acid or cysteine.   Since the hydrolysis of this tripeptide involves the scission of two peptide linkages, a quantitative study of the kinetics of its hydrolysis is more complex than in the case of l-leucylglycine.   However, it has been observed that as a result of dialysis against distilled water the activity toward l-leucylglycylglycine in the presence of cysteine or ascorbic acid is diminished, so that the possibility exists that Cathepsin III also performs the hydrolysis of this tripeptide.

The fact that a portion of the enzymatic activity of beef spleen cathepsin is lost on dialysis against distilled water is at present the subject of further investigation.   In particular, it will be of interest to determine whether the inactivation is due to the removal of a dialyzable component or to the destruction of the enzyme protein.

Further Characterization of Cathepsin I—It was noted earlier

[2] After a 24 hour dialysis against distilled water, the activity in the presence of ascorbic acid was too low to be measured accurately.

that Cathepsin I may be inactivated at 40°. This sensitivity to heat has been employed to demonstrate the distinct nature of Cathepsin I. When a solution of beef spleen cathepsin was heated at 50° for 10 minutes, a precipitate appeared. The clear filtrate was tested for enzymatic activity toward carbobenzoxy-l-glutamyl-l-tyrosine, benzoyl-l-arginineamide, and l-leucineamide. The results in Table VI show that while the cysteine-activatable en-

TABLE V

Hydrolysis of l-Leucineamide and l-Leucylglycine by Beef Spleen Cathepsin

Solution A of beef spleen cathepsin was dialyzed against distilled water for 2 hours at 4° in a Kunitz dialyzer. The flocculent precipitate was filtered off and the clear filtrate, Solution B, was employed for hydrolytic experiments. Another portion of Solution A was heated at 65° for 10 minutes. The flocculent precipitate was filtered off and the clear filtrate, Solution C, was employed for hydrolytic experiments   Temperature of hydrolytic experiments, 40°.

Enzyme solution	Protein N per cc. test solution	Activator	l-Leucineamide*		l-Leucylglycine†		$\dfrac{c_{LA}(Cy)}{c_{LA}(Asc)}$	$\dfrac{c_{LA}(Cy)}{c_{LG}(Cy)}$
			K (first order)	c_{LA}	K (first order)	c_{LG}		
	mg.							
A	0.36	Cysteine	0.0032	0.0089	0.0016	0.0045		2.0
		Ascorbic acid	0.00070	0.0020	0.00039	0.0011	4.5	
B	0.39	Cysteine	0.0012	0 0031	0.00071	0.0018		1.7
		Ascorbic acid	0.00025	0.00064	0.00015	0.00039	4.8	
C	0.23	Cysteine	0.0013	0.0058	0.00075	0.0032		1.8
		Ascorbic acid	0.00028	0.0012	0.00020	0.00087	4.8	

* pH 5.2.
† pH 5.0.

zyme activity toward benzoyl-l-arginineamide and l-leucineamide remained unaffected by this treatment, the reaction velocity constant for the splitting of carbobenzoxy-l-glutamyl-l-tyrosine decreased to two-thirds of its original value. These results indicate that Cathepsin I is an enzymatic component of beef spleen extracts different in nature from Cathepsin II or III. Furthermore, it will be noted that the rate of splitting of carbobenzoxy-l-glutamyl-l-tyrosine by the heat-treated solution is the same whether or not

cysteine had been added; this serves as added proof that the activity of Cathepsin I is not increased by the addition of cysteine.

In a previous publication (2) it was reported that the substrate carbobenzoxy-*l*-glutamyl-*l*-phenylalanine was hydrolyzed appreciably by beef spleen cathepsin in the absence of cysteine but that on addition of cysteine, a marked increase in the rate of splitting was observed. The hydrolysis of this substrate has been reinvestigated in the light of the above results.

When carbobenzoxy-*l*-glutamyl-*l*-phenylalanine was subjected to the action of unactivated beef spleen cathepsin at 25°, first order kinetics were observed. It will be noted that the rate of

TABLE VI

Heat Inactivation of Cathepsin I

Solution A of beef spleen cathepsin (2.83 mg. of protein N per cc. of enzyme solution) was heated at 50° for 10 minutes and then was chilled in ice water. The flocculent precipitate that appeared was filtered off and the clear filtrate, Solution B, was employed for the hydrolytic experiments. 0.3 cc. of the enzyme solutions was added per 2.5 cc. of reaction mixture.

Substrate	Activator	pH	Temperature	K (first order)	
				Enzyme Solution A	Enzyme Solution B
			°C.		
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	None	5.3	25	0.0012	0.0008
	Cysteine	5.3	25	0.0013	0.0008
Benzoyl-*l*-arginineamide	"	4.9	40	0.0028	0.0027
l-Leucineamide	"	5.2	40	0.0033	0.0033

hydrolysis of this substrate is slower than that for carbobenzoxy-*l*-glutamyl-*l*-tyrosine (Table VII). When the splitting of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine was performed in the presence of cysteine, a more rapid hydrolysis was observed. The hydrolysis did not proceed further than the scission of one peptide linkage per substrate molecule. Furthermore, carbobenzoxy-*l*-glutamic acid and *l*-phenylalanine were isolated from the reaction mixture (*cf.* the experimental section).

Parallel experiments were performed in which carbobenzoxy-*l*-glutamyl-*l*-phenylalanine as well as carbobenzoxy-*l*-glutamyl-*l*-tyrosine was subjected to the action of an unactivated beef spleen extract that had been heated at 50° for 30 minutes. In Table VII the hydrolytic constants thus obtained are compared

with the corresponding constants obtained with the unheated spleen extract. It will be noted that as a result of the heat treatment the constants for both substrates decreased to the same degree; *i.e.*, 66 and 64 per cent. This indicates that in the ab-

TABLE VII

Hydrolysis of Carbobenzoxy-l-Glutamyl-l-Phenylalanine* by Beef Spleen Cathepsin

Solution A of beef spleen cathepsin (2.83 mg. of protein N per cc. of enzyme solution) was heated at 50° for 30 minutes and then was chilled in ice water. The flocculent precipitate that appeared was filtered off and the clear filtrate, Solution B, was employed for the hydrolytic experiments. 0.4 cc. of the enzyme solutions was added per 2.5 cc. of reaction mixture.

Substrate	Activator	Time	Enzyme Solution A		Enzyme Solution B	
			Hydrolysis	K	Hydrolysis	K
		min.	*per cent*		*per cent*	
Carbobenzoxy-*l*-gluta-	None	120	18	0.00072	8	0.00025
myl-*l*-phenylalanine†		240	33	0.00073	13	0.00025
		360	44	0.00070	21	0.00028
	Cysteine	120	38		28	
		240	62		43	
		360	77		56	
		480	92			
		1440	97			
Carbobenzoxy-*l*-gluta-	None			0.00147		0.00050
myl-*l*-tyrosine†						
Benzoyl-*l*-arginineamide‡	Cysteine			0.0033		0.0030
l-Leucineamide§	"			0.0041		0.0037

* The preparation of this substrate has been described in a previous paper (11).

† pH 5.3; temperature 25°.

‡ pH 4.9; temperature 40°.

§ pH 5.2; temperature 40°.

sence of cysteine the hydrolysis of these two substrates is performed by the same enzyme; namely, Cathepsin I.[3]
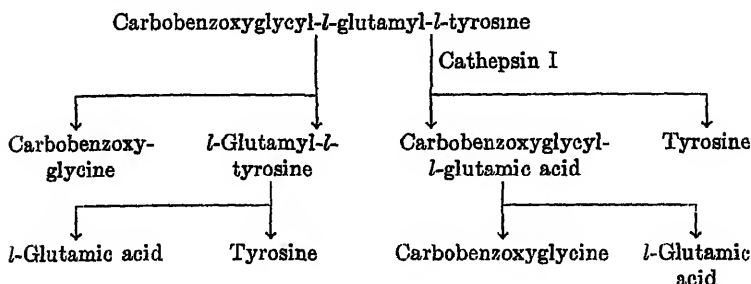
[3] Benzoyl-*l*-arginineamide and *l*-leucineamide have been shown to be split in the presence of cysteine by Cathepsins II and III respectively. Both these enzymes are less heat-sensitive than Cathepsin I, and as a result of the heating only about 10 per cent of their activity was lost (Table VII).

When the hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-phenylala-
nine by the heated spleen extract was performed in the presence
of cysteine, the rate was much greater than in the absence of
cysteine.   It will be recalled that a similar increase was observed
for the hydrolysis of this substrate by the beef spleen extract be-
fore heating at 50°.   Since it has previously been shown that
Cathepsin I is not activated by cysteine, it follows that an addi-
tional enzyme besides Cathepsin I participates in the hydrolysis
of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine by beef spleen ca-
thepsin.   This presents an interesting example of a substrate that
is hydrolyzed at the same peptide linkage by two different en-
zymatic components of beef spleen, one of which is activated by
cysteine while the other is not activated by cysteine.   At present it
has not been established whether or not the cysteine-activatable
enzyme that splits carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is
identical with Cathepsin II.

Another example of a substrate hydrolyzed both by Cathepsin
I and another component of beef spleen cathepsin is found in the
compound carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine (11).   This
substrate is split in the absence of cysteine by Cathepsin I at
25° with a first order velocity constant of 0.00057 and yields ty-
rosine as a hydrolytic product.   (The other split-product, car-
bobenzoxyglycyl-*l*-glutamic acid, is not measurably hydrolyzed
under these conditions.)   When the carbobenzoxyglycyl-*l*-glu-
tamyl-*l*-tyrosine was subjected to the action of the enzyme solu-
tion that had been heated to 50° for 30 minutes, the first order
velocity constant dropped to 0.00022, representing a loss of 61
per cent of the original activity.   Since this heat treatment had
resulted in a decrease of 66 per cent in the rate of splitting of car-
bobenzoxy-*l*-glutamyl-*l*-tyrosine by Cathepsin I (*cf*. Table VII),
it may be inferred that the hydrolysis of the linkage between the
glutamyl and tyrosine residues in carbobenzoxyglycyl-*l*-glutamyl-
l-tyrosine was performed by Cathepsin I.

It was previously found that the addition of cysteine markedly
accelerates the hydrolysis of carbobenzoxyglycyl-*l*-glutamyl-*l*-
tyrosine (2).   Preliminary experiments have shown that this in-
crease in rate is due to the action of components of beef spleen
cathepsin other than Cathepsin I.   The course of the hydrolysis
of carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine by cysteine-activated

cathepsin is rather complex and may be represented by the accompanying diagram.

Carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine

|Cathepsin I

Carbobenzoxy-glycine   *l*-Glutamyl-*l*-tyrosine   Carbobenzoxyglycyl-*l*-glutamic acid   Tyrosine

l-Glutamic acid   Tyrosine   Carbobenzoxyglycine   *l*-Glutamic acid

Two of the final split-products, carbobenzoxyglycine and tyrosine, have been isolated and characterized. Furthermore, it has been found that, in the initial stages of hydrolysis of carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine by cysteine-beef spleen cathepsin, the liberation of amino nitrogen proceeds much more rapidly than the liberation of amino acid carboxyl groups (12).[4] This may be taken as evidence that the substrate is hydrolyzed at the linkage between the glycyl and glutamyl residues. Finally, the two intermediary products, *l*-glutamyl-*l*-tyrosine and carbobenzoxyglycyl-*l*-glutamic acid, have been found to be hydrolyzed by cysteine-beef spleen cathepsin.

The number of simultaneous and successive reactions that may occur when a substrate with only two sensitive peptide linkages is subjected to the action of a tissue extract emphasizes the caution with which data obtained by means of much more complex substrates, such as proteins, must be treated.

It has frequently been found that an enzyme previously regarded as an individual has later been shown to consist of several enzymatic components. However, until evidence of such enzymatic inhomogeneity is observed, it is necessary to consider such an enzyme as a single, distinct enzyme. It is intended to regard each of the three spleen cathepsins characterized in this paper as enzymatic individuals until indication of enzymatic inhomogeneity is found. It may also be pointed out that the identification of three

[4] We wish to thank Dr. Alma Hiller for kindly performing the carboxyl determinations.

distinct enzymes in beef spleen extracts was made possible through the use of only a few synthetic substrates. It seems likely that the application of a greater number of synthetic substrates may reveal the presence of additional enzymes in beef spleen.

It has been pointed out that Cathepsin I does not require the addition of an activator, is extremely heat-sensitive, and becomes less sensitive when cysteine is added. In these properties Cathepsin I bears some resemblance to the proteinase component of beef spleen cathepsin studied by Anson (4). The characterization of Cathepsin II shows that a proteinase which requires activation is also present in beef spleen. Cathepsin II may be classified as an endopeptidase (13). Cathepsin III may be tentatively classified as an exopeptidase (13).

EXPERIMENTAL

The beef spleen cathepsin solutions employed in this publication were prepared as described in a previous paper (2). In following the course of enzymatic hydrolysis, the microtitration method of Grassmann and Heyde was employed for all the substrates with the exception of carbobenzoxy-*l*-glutamyl-*l*-tyrosine, when the hydrolysis was followed by amino nitrogen determinations. The Grassmann-Heyde method does not give completely satisfactory end-points with tyrosine-containing substrates. The substrate concentration was 0.05 mM per cc. of test solution in all cases The pH was adjusted by means of citrate buffers.

Cysteine or ascorbic acid was employed as the activator in a concentration of 0.01 mM per cc. of test solution.

Isolation of Split-Products from Hydrolysis of Carbobenzoxy-l-Glutamyl-l-Phenylalanine by Cysteine-Beef Spleen Cathepsin—The enzymatic hydrolysate of 428 mg. of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine was concentrated to a small volume and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate layer was washed with water and then extracted with dilute bicarbonate solution. The bicarbonate extract was then acidified to yield a syrup that crystallized on standing in the ice chest. The dry material (197 mg.) melted at 119° and gave

a mixed melting point of 119° with an authentic sample of car-
bobenzoxy-*l*-glutamic acid.

Carbobenzoxy-l-Glutamic Acid—

$C_{13}H_{15}O_6N$.   Calculated.   C 55.5, H 5.4, N 5.0

281.2   Found.   " 55.7, " 5.5, " 5.2

The acid aqueous layer that had been extracted with ethyl
acetate was concentrated to 4 cc.   This solution was heated and a
solution of 350 mg. of naphthalene-β-sulfonic acid in 1 cc. of water
was added.   The crystals that separated on standing in the ice
chest were filtered, washed with ice water, and dried.   This ma-
terial (287 mg.) was recrystallized from hot water.

l-Phenylalanine Nasylate (14)—

$C_9H_{11}O_2N \cdot C_{10}H_8O_3S$.   Calculated.   C 61.1, H 5.1, N 3.8

373.4   Found.   " 61.0, " 5.1, " 4.0

SUMMARY

Through the study of the kinetics of the action of beef spleen
cathepsin on several synthetic substrates it has been possible to
characterize three distinct enzymes designated Cathepsins I, II,
and III.   Cathepsin I hydrolyzes carbobenzoxy-*l*-glutamyl-*l*-ty-
rosine and does not require the addition of an activator such as
cysteine.   Cathepsin II hydrolyzes benzoyl-*l*-arginineamide when
an activator such as cysteine has been added.   Cathepsin III
hydrolyzes *l*-leucineamide and *l*-leucylglycine in the presence of
activators such as cysteine or ascorbic acid.

BIBLIOGRAPHY

1. Bergmann, M., and Niemann, C., *Science*, **86**, 187 (1937).   Bergmann,
   M., *Chem. Rev.*, **22**, 423 (1938).   Fruton, J. S., in Cold Spring Harbor
   symposia on quantitative biology, Cold Spring Harbor, **6**, 50 (1938).
2. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130**, 19 (1939).
3. Rondoni, P., *Klin. Woch.*, **17**, 1601 (1938).
4. Anson, M. L., *J. Gen. Physiol.*, **23**, 695 (1940).
5. Willstatter, R., and Bamann, E., *Z. physiol. Chem.*, **180**, 127 (1928).
6. Waldschmidt-Leitz, E., Schäffner, A., Bek, J. J., and Blum, E., *Z.
   physiol. Chem.*, **188**, 17 (1930).
7. Grassmann, W., Dyckerhoff, H., and Schoenebeck, O., *Z. physiol.
   Chem.*, **186**, 183 (1929).
8. Bergmann, M., Zervas, L., Salzmann, L., and Schleich, H., *Z. physiol.
   Chem.*, **224**, 17 (1934).

9. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).
10. Behrens, O. K., and Bergmann, M., *J. Biol. Chem.*, **129**, 587 (1939).
11. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **127**, 627 (1939).
12. Van Slyke, D. D., and Dillon, R. T., *Proc. Soc. Exp. Biol. and Med.*, **34**, 362 (1936).
13. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117**, 189 (1937).
14. Bergmann, M., and Stein, W. H., *J. Biol. Chem.*, **129**, 609 (1939).

A FIBROUS PROTEIN FROM THE SLIME OF THE HAGFISH

By JOHN D. FERRY*

(From the Department of Physical Chemistry, Harvard Medical School, Boston, and the Hopkins Marine Station of Stanford University, Pacific Grove, California)

The hagfishes are well known for the copious amounts of slime, or "mucus," which they secrete when disturbed or annoyed (1). In sea water, a hagfish can produce in a few seconds several times its own volume of slime. The slime is colorless and transparent, and, although extremely dilute, has a tough, coherent, stringy consistency. It is highly viscous, and also possesses rigidity, exhibiting "elastic recoil." This material, obtained from the Pacific hagfish, *Polistotrema stouti*, has now been examined in connection with a general study of mechanical properties of protein, mucoprotein, and polysaccharide systems. It has been found to be heterogeneous in composition, containing insoluble protein fibers which are largely responsible for its peculiar consistency. The present paper describes the microscopic structure of the slime and the composition of the fiber protein.

Materials—Specimens of *Polistotrema stouti* were obtained from Monterey Bay. The animals survived for several weeks without food in tanks of running sea water, and could be stimulated repeatedly for the production of slime. I am greatly indebted to Dr. Berry Campbell for advice and aid in the handling of hagfish and in devising suitable methods of stimulation.

Behavior of Slime Secreted in Sea Water—The animals were stimulated in sea water by agitation or local pressure. The voluminous transparent slime thus produced contained a loosely tangled mass of fibers, each about 1 3 μ in diameter and several cm long. This mass later contracted spontaneously and ir-

---

reversibly, expelling water, to about a fiftieth of its original volume. From the resulting compact mat of fibers, strands could be drawn several feet in length and variable in thickness (0 05 to 0 5 mm). The strands were soft and elastic when wet, and strong and flexible when dry.   The yield of fibers was about 0.25 gm. from a liter of slime.   The sea water expelled from the original slime contained about 0.01 per cent of a dissolved protein which could not be precipitated by ammonium sulfate or trichloroacetic acid, but could be recovered in a denatured form by dialysis, electrodialysis, and evaporation to dryness

FIG 1 Secretion from the slime gland, diluted somewhat with water, showing fiber coils (about 0.11 mm in length).

Behavior of Slime Secreted in Absence of Water; Fiber Coils—In order to avoid the presence of a large excess of sea salt, as well as occasional particles of foreign matter present in sea water, the secretion was also obtained undiluted by stimulating the animals out of water   The hagfish was anesthetized with ether, suspended vertically, and wiped clean.   Upon local electrical stimulation, the slime glands discharged white drops of secretion.   The latter contained no extended fibers, but fibers in tightly rolled coils of uniform elliptical shape about 0 11 mm. in length and 0.06 mm. in width (Fig. 1).   Several dozen of these coils were discharged simultaneously from a single duct, as could be observed with the microscope.   When the secretion was diluted with either sea water or distilled water, and slightly agitated, the coils unrolled

to produce extended fibers (Figs. 2 and 3) and the suspension acquired the characteristic appearance and consistency of the slime as ordinarily secreted in water.

Most of the coils unrolled immediately, but some remained coiled and could not be unrolled even by vigorous agitation. When secretion took place directly in water, on the other hand, the unrolling was almost complete, although microscopic examination of the slime revealed an occasional intact coil.[1]

FIG. 2   FIG. 3

FIG 2 Fiber coil unrolling upon agitation.

FIG. 3. Fibers (about $1\,\mu$ in diameter) almost completely unrolled, forming a concentrated slime.

Isolation of Soluble Protein from Slime Secretion—Several hagfish were stimulated electrically and the drops of secretion washed down with distilled water, producing a concentrated slime, which was filtered on a Buchner funnel. The filtrate, acidified with acetic acid, formed a thixotropic gel. Upon dilution with several volumes of alcohol, the gel shrank; it was then repeatedly washed with alcohol and finally dried. The yield was very small, amounting to about 5 per cent of the accompanying fiber protein. The

[1] The mechanism of this curious process of secretion of fibers in preformed coils is being investigated by Professor W W. Newby of the University of Utah, who is studying the histology of the slime glands

nitrogen content of this soluble material (by digestion and nesslerization) was 12.7 per cent and the carbohydrate content[2] (as glucose, by the method of Sørensen and Haugaard (2)) was 2.9 per cent. The dried protein swelled enormously in water, and was dissolved by treatment with ascorbic acid-hydrogen peroxide, suggesting that its rigidity might be associated with a phosphoric acid component.[3]

Purification of Fiber Protein—The mat of fibers collected from the filtration above was resuspended in distilled water, stirred thoroughly, and then run through rubber rollers and squeezed dry The resulting films were cut in small pieces and washed twice by soaking an hour in distilled water and squeezing with a stirring rod. They were finally washed with alcohol and ether and dried, yielding a fluffy mass of pure white fibers. The ash content of this material, estimated by igniting with sulfuric acid at 500°, was 1 1 per cent. A portion was later suspended in phosphate buffer (ionic strength 0.2) at pH 7 7 to dissolve possible traces of remaining soluble protein, washed phosphate-free, electrodialyzed, and redried with alcohol and ether. The fiber protein thus purified had an ash content of 0.9 per cent, and contained 17.5 per cent nitrogen (corrected for ash) and 1.4 per cent carbohydrate.

The tyrosine content was estimated by the method of Lugg (3). Duplicate samples were hydrolyzed in 3.3 N sodium hydroxide. Values of 5.3 and 5.6 per cent tyrosine were obtained. The tryptophane content, according to Lugg's method, was less than 0 2 per cent. The cystine content was estimated by the method of Folin and Marenzi (4). Duplicate samples were hydrolyzed in 6 N sulfuric acid; the humin formation was so slight that no decolorizing was necessary Values of 0.38 and 0 46 per cent cystine were obtained No great accuracy is claimed for these analytical figures, in the absence of parallel analyses upon proteins of known composition by the same procedures, especially in hydrolysis. However, they serve to characterize the composition of the fibers, and to differentiate them clearly from other fibrous proteins.

The fibers were insoluble in dilute acid, alkali, and salt solu-

[2] I am much indebted to Miss Sue Y. Green for carbohydrate analyses.

[3] Robertson, W. van B , personal communication.

tions, and in 6 M urea. In any of these solutions they softened and formed a swollen mat, from which strands could be drawn; but the high dilution and characteristic properties of the original slime were not regained. Upon being autoclaved with water at 120° for 4 hours, the substance was still undissolved, but its tensile strength was much impaired.

DISCUSSION

The composition of the fibrous protein from hagfish slime may be compared with that of collagen, myosin, fibroin, and the keratins (5). The absence of tryptophane, within the limits of the analysis, and the low cystine content distinguish the protein from myosin and the keratins. Its moderately high tyrosine content differentiates it from collagen, assuming the composition of the latter to be similar to that of its derivative gelatin, which is tyrosine-free. On the other hand, it contains much less tyrosine than does silk fibroin. Thus it appears to be quite unlike any of these fibrous proteins. The name *mitin* ($\mu\iota\tau\sigma s$, thread) is suggested for the fiber protein from *Polistotrema* and for similar substances which may subsequently be obtained from related animals. It will be of interest to compare the configuration of mitin as studied by x-ray diffraction[4] with similar results for natural and derived protein fibers (6).

The heterogeneity of the original slime and its irreversible contraction render it unsuitable for study of mechanical properties in relation to its composition and structure. However, it might serve as a large scale model to illustrate structures which have been proposed for certain gels with no optically visible structure. It consists of a tangle of threads, whose ratio of length to diameter is of the order of 10^5, and whose surfaces are lyophilic in character, suspended in a medium of relatively low viscosity. Shear is accompanied by straightening and aligning the tangled threads to some extent, while recovery from shear ("elastic recoil") involves resumption of the random entanglement. This is not unlike the supposed molecular structure of a gel of rubber, or better, polyisobutylene or polystyrene (in which there are no cross links joining

[4] Dr. R. B. Corey of the California Institute of Technology is investigating the x-ray diffraction of mitin fibers, and has observed patterns which differ from those of other fibrous proteins.

the molecular chains), swollen in a non-polar solvent (7).   On the other hand, the unrolling of coils of mitin, with the formation of an elastic slime, might be a model for the denaturation of a globular protein (6), which under certain conditions transforms a solution of low viscosity into a rigid gel.

The author is much indebted to Professors W. K. Fisher and C. B. van Niel and their associates for the facilities of the Hopkins Marine Station.

SUMMARY

The slime of the hagfish contains a fibrous protein secreted in the form of coils which unroll to form extended fibers.   The composition and properties of this protein, which differ widely from those of other fibrous proteins, are described.

BIBLIOGRAPHY

1. Parker, T. J., and Haswell, W. A., Text-book of zoology, London, 2, 138 (1910).
2. Sørensen, M., and Haugaard, G., *Compt.-rend. trav. Lab. Carlsberg*, 19, No. 12 (1933).
3. Lugg, J. W. H., *Biochem. J.*, 31, 1422 (1937); 32, 775 (1938).
4. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 83, 103 (1929).
5. Cohn, E. J., *Chem. Rev.*, 24, 203 (1939).
6. Astbury, W. T., *Ann. Rep. Progress Chem.*, 35, 198 (1938); in Luck, J. M., Annual review of biochemistry, Stanford University, 8, 113 (1939).
7. Sparks, W. J., Lightbown, I. E., Turner, L. B., Frohlich, P. K., and Klebsattel, C. A., *Ind. and Eng. Chem.*, 32, 731 (1940).   Whitby, G. S., *Tr. Inst. Rubber Ind.*, 6, 55 (1930); *J. Physic. Chem.*, 36, 198 (1932).

DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE BLOOD CELLS OF VARIOUS VERTEBRATES

By S. RAPOPORT and GEORGE MARTIN GUEST

(From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)

This paper presents data on the distribution of acid-soluble phosphorus compounds in the blood cells of a few apparently normal individuals of each of forty-six species: twenty-two mammalian, twelve avian, six reptilian, four amphibian, and two fish. Since the studies were limited by availability of material, each order is represented by only a few species, and in many instances only one blood sample from a species could be examined. Little consideration could be given to the possible influence of age, sex, seasonal variation, diet, physiologic state of the individual, and other factors known to affect the concentration of phosphorus in the bloods of certain animals which have been studied more extensively. Although incomplete in these respects, the data permit a general description of gross differences in the concentrations and relative distribution of these phosphorus compounds found in the bloods of the several species examined.

Such data on the distribution of phosphorus compounds in the bloods of the different species are of considerable interest in connection with evolutionary relationships among the species, a field of study which has been little explored. Kay (1) determined the distribution of phosphorus, including fractionation of the organic acid-soluble P by hydrolysis with bone enzyme, in the bloods of eleven mammalian species and discussed possible relationships which might exist between the phosphorus content and other properties of the cells. His data on the fraction hydrolyzable by bone enzyme correspond roughly with values for adenosine triphosphate, determined by other methods. Kerr and Daoud (2) in 1935 reported data on the concentrations of inorganic P, adenosine triphosphate, and organic acid-soluble P in the bloods

of twenty-four species, and later Kerr (3) added similar data on four additional species. In a study primarily concerned with fish, McCay (4) reported data on the acid-soluble P content of the bloods of three lower vertebrates. The normal distribution of inorganic P, organic acid-soluble P, adenosine triphosphate, and diphosphoglycerate in the bloods of man, dog, rabbit, and rat has been described in a series of papers from this laboratory dealing with studies of blood phosphorus in various conditions (5).

In the early stages of the studies here reported, it was noted that diphosphoglycerate, which makes up about half of the organic acid-soluble P in most mammalian bloods, was absent in the bloods of all birds. The finding of a high concentration of organic acid-soluble P in the bird bloods led to a search for a previously unidentified substance which proved to be phytic acid (6). The subsequent development of a suitable method for the determination of phytic acid in blood permitted the collection of quantitative data on the concentration of this substance in avian blood corpuscles.

Methods

Blood samples were obtained from some of the less common species of animals at the Cincinnati Zoological Garden, through the cooperation of Dr. Sol Stephens. Fish were obtained from the Ohio State Fish Hatchery at Newtown, Ohio, with the permission of Dr. T. H. Langlois. The more common fowls were purchased in the market. The amphibia were shipped from New Orleans.

Most of the animals were bled in the laboratory by vein, artery, or heart puncture, a needle and a glass syringe being used. Mice were bled by cutting the jugular vein, and bloods from ten mice were pooled for each set of analyses. Reptiles, amphibia, and fish were bled by heart puncture. Turtle blood was collected by heart puncture or by cutting one carotid artery. Birds were usually bled from a wing vein. Pig, sheep, and beef bloods were obtained at the slaughter-house, defibrinated, and transported to the laboratory as quickly as possible, usually within 15 minutes.

Except when defibrinated, the bloods were collected in vials containing 1 mg. of phosphorus-free heparin per 5 cc. of blood. For deproteinization, in most instances the blood (usually 5 cc.)

was precipitated with an equal volume of 20 per cent trichloro-acetic acid, and 3 volumes of distilled water were added. After thorough shaking, the mixture was allowed to stand 5 minutes and then filtered. The minute amounts of phosphoglyceric acid in beef, sheep, and goat bloods necessitated the use of larger amounts of blood. Therefore 50 cc. portions of these bloods were precipitated with an equal volume of 20 per cent trichloroacetic acid, and 1 volume of water was added. For the deproteinization of all bloods containing nucleated erythrocytes, 1 volume of blood was precipitated with 2 volumes of 20 per cent trichloroacetic acid, and 2 volumes of water were added. This higher concentration of trichloroacetic acid was necessary to avoid the development of turbidity when ammonium molybdate solution was added for the determination of inorganic P.

In order to have sufficient material to test for phosphoglyceric acid in bloods containing nucleated erythrocytes, from 10 to 40 cc. of avian blood were precipitated in the manner just described. In order to test for phytic acid in most of the reptilian and amphibian bloods, which had a low volume of cells, the cells were separated from the plasma by centrifugation and then precipitated with trichloroacetic acid as described above. The other phosphorus fractions in these bloods were, however, determined on whole blood, because centrifugation and packing of the cells permit the decomposition of adenosine triphosphate. The concentrations of the several fractions in the cells were calculated, from values determined on whole blood, by means of the per cent *total* cell volume; *i.e.*, including the white cells. No attempt was made to analyze the erythrocytes and leucocytes separately.

The volume of packed cells was determined by the method of Guest and Siler (7). Inorganic and total acid-soluble P was determined by the method of Fiske and Subbarow (8) with minor modifications. The pyrophosphate fraction was determined by hydrolysis of the trichloroacetic acid filtrates in N H_2SO_4 for 8 minutes in a boiling water bath, and the pyrophosphate values were multiplied by 3/2 to obtain the adenosine triphosphate P. The validity of this calculation is supported by the findings of Kerr and Daoud (2), who determined simultaneously the pyrophosphate and nucleotide P in the bloods of twenty-four species and found that in most species the values of the two fractions

approached the theoretical ratio of 2:1. Phosphorylated glyceric acid was determined by the method of Rapoport (9), with varying aliquots of the filtrates from blood samples of different species. From bloods rich in phosphoglycerate, amounts of filtrate equivalent to 2 cc. of blood and, from other bloods, amounts of filtrate corresponding to 2 to 6 cc. of blood were used. In the test for phosphoglycerate, all samples were inspected after being heated with the naphthoresorcinol reagent before they were diluted for colorimetric comparison. If no trace of blue or green color was seen, the qualitative test for glyceric acid was recorded as negative. Negative tests for phosphoglycerate were thus obtained with all bloods containing nucleated erythrocytes. Unknown substances in beef, sheep, and goat bloods gave rise to interfering brown colors. Therefore the figures for phosphoglycerate in those bloods, as listed in Table I, probably are higher than the true values. The determination of phytic acid was made by the method of Michel-Durand (10), modified by Leva and Rapoport (unpublished work). For the determination of phytic acid in bird bloods, amounts of trichloroacetic acid filtrate equivalent to 2 cc. of blood were used. For reptilian and amphibian bloods, amounts of filtrate corresponding to 2 cc. of cells were used. Except turtle blood, all bloods examined other than those of birds failed to show the presence of phytic acid.

Results

Mammals (Table I)—The twenty-two species, belonging to seven orders, represented in Table I may be divided into two main groups according to the concentrations of organic acid-soluble P compounds in the blood cells.

In one group which includes most of the mammals studied the concentration of organic acid-soluble P in the blood cells varied between 50 and 100 mg. per 100 cc. In most instances about half of the organic acid-soluble P was accounted for as diphosphoglycerate, and from 15 to 25 per cent as adenosine triphosphate. In horse blood, the proportion of diphosphoglycerate was higher, and that of adenosine triphosphate was lower, than in most of the other bloods of this group. According to the analyses of Kerr and Daoud (2, 3), the bloods of the wolf, jackal, mule, mole-rat, and ass would also fall into this group. The concentration of

TABLE I

Distribution of Acid-Soluble P in Bloods of Twenty-Two Mammalian Species

Species	Whole blood		Cells			Proportions of organic acid-soluble P in	
	Volume of packed cells	Inorganic P	Adenosine tri-phosphate P	Diphospho-glycerate P	Organic acid-soluble P	Adenosine tri-phosphate	Diphospho-glycerate
	per cent	mg. per 100 cc.	mg per 100 cc.	mg per 100 cc.	mg. per 100 cc.	per cent	per cent
Pig, *Sus scrofa*	40.3	7.3	20.8	43.7	98 7	21	44
" " "	42 0	6 9	22.4	45.0	95.0	24	47
Armadillo, *Dasypus sixcinctus*	34.3	4.6	43.7	26.6	96.7	45	28
Rabbit, *Lepus cuniculus**	39.6	4 5	21.1	45 3	87.9	24	51
Mouse, *Mus musculus*†	41.1	7.4	12.1	51.8	84 1	14	62
" " "	38 5	7.9	16.0	54.0	85.8	19	63
Rat, *Mus norvegicus albinus**	40.8	5 6	14.4	34.1	66.8	22	51
Raccoon, *Procyon lotor*	36.1	4.3	4.0	27.4	61.6	7	45
Guinea pig, *Cavia domestica*	32 7	5.1	11 2	34.8	59.7	19	58
" " " "	47 7	4.6	10.8	28.4	49.4	22	57
" " " "	36 0	4.9	10.4	30 1	47.6	22	63
Monkey, *Macacus rhesus**	39 9	3 6	13 4	31.0	56.6	24	55
" " cynomolgus	42.2	3.1	11 3	28.9	47 0	24	61
" " "	45.6	4 1	11.4	32.0	49.3	23	65
Man, *Homo sapiens**	43 5	3.2	13.5	28 0	55.0	24	51
Dog, *Canis familiaris**	49.8	3 2	9 9	31 0	52.1	19	60
Camel, *Camelus bactrianus*	34 8	5 6	10 9	29 1	54.1	20	54
Kangaroo, *Macropus rufus*	49.9	4 3	9.2	18.6	53 1	17	35
Horse, *Equus caballus*	32.9	2.1	3.7	33.4	50 9	7	66
" " "	28 1	2 2	3 2	31.5	44.9	7	70
Opossum, *Didelphys virginiana*	42 9	3.3	12 1	22.8	50.9	24	45
Elephant, *Elephas indicus*	32.2	3.4	12 5	18 7	47 9	26	39
Indian dwarf ass, *Equus asinus*	43 4	2.0	3 0	15.6	30.9	10	51
Cat, *Felis domestica*	28.9	5.0	9 4	3.5	20.2	47	17
" " "	29.1	4.4	10 2	4.4	19.4	53	22
Sheep, *Ovis aries*	35.0	6 5	8.4	<0.8	15.4	54	5
Karakul sheep, *Ovis platyora*	46.8	3.3	6 5		10.0	65	
Sambar deer, *Cervus hippelaphus*	32 4	4 2	6.1		11.6	53	
Goat, *Capra hircus*	26 7	6.8	7.3	<0.2	11.8	62	2
" " "	28.7	8.4	7 3	<0.2	10.8	67	2
Beef, *Bos taurus*	45.7	8.5	6.0	<0.7	10.8	55	6
" " "	45.2	6.7	4.2	<0 4	8.7	48	5

* Average values for each species, twelve rabbits, eleven rats, six monkeys, twenty humans, thirteen dogs.

† Pooled sample of bloods from ten mice.

organic acid-soluble P in the blood cells of the dwarf Indian ass reported here was somewhat less than was found in the blood of the ass by Kerr. While this may be a difference between two subspecies, it should be noted that this was an old animal which had been kept in the Cincinnati zoo some 20 years. The low concentration of inorganic P suggests that this animal may have had a mild phosphorus deficiency.[1]

Another group of mammals, including sheep, the sambar deer, goats, and beef, had comparatively low concentrations of organic acid-soluble P in the blood cells, varying from 9 to 15 mg. per 100 cc. Very small amounts of phosphoglycerate were found in these bloods, and it is uncertain whether this occurred as the mono- or diphosphoglyceric acid. Findings which will be reported later suggest that it is the monophosphoric ester that is present in these bloods. Calculated as such, less than 6 per cent of the organic acid-soluble P in these bloods can be accounted for in this fraction. The adenosine triphosphate comprised around 50 per cent or more of the organic acid-soluble P in these bloods, leaving approximately 40 per cent unidentified. According to the analyses of Kerr and Daoud (2, 3), the bloods of the buffalo, gazelle, and hyena would fall into this group.

The bloods of the cat and armadillo fall into neither of these two general groups. The blood cells of the domestic cat contained around 20 mg. of organic acid-soluble P per 100 cc., of which approximately 20 per cent was accounted for as phosphoglycerate (calculated as diphosphoglycerate). In the blood cells of the armadillo the concentration of organic acid-soluble P was very high, with a comparatively low concentration of phosphoglycerate and the highest concentration of adenosine triphosphate found among the mammalian bloods.

---

[1] In the evaluation of data such as are reported here, it must be borne in mind that unsuspected phosphorus or vitamin D deficiency may account for low concentrations of organic acid-soluble P as well as of inorganic P in the blood. For example, we found this unexpectedly in the bloods of some monkeys which had been kept confined several months in cages away from sunlight. In these monkeys the addition of vitamin D to the diet, without other change, was followed by increases in the concentration of organic acid-soluble P in the blood cells to normal levels. Decreased concentrations of organic acid-soluble P (both adenosine triphosphate and diphosphoglycerate) in the blood cells of rats receiving a rachitogenic high Ca-low P diet have been reported previously (5).

Birds (Table II)—In the bloods of twelve avian species, belonging to seven orders, the concentration of organic acid-soluble

TABLE II

Distribution of Acid-Soluble P in Bloods of Twelve Avian Species

Species	Whole blood				Cells		Proportions of organic acid-soluble P in	
	Volume of packed cells	Inorganic P	Adenosine triphosphate P	Phytic acid P	Organic acid-soluble P		Adenosine triphosphate	Phytic acid
	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		per cent	per cent
Pigeon, *Columba livia*	34 2	6.8	39.7	86.8	134.1		30	65
" " "	52.5	6.5	35.8	71.5	120.2		30	59
" " "	47.8	5.2	30.6	71.6	115 0		27	62
" " "	46.0	7.1	31.3	67.4	110.6		28	61
Turkey, *Meleagris gallopavo*	31.9	5.2	16.8		135.4		13	
" "	41.2	6.0	16.2	74.2	112.5		15	66
Canary, *Serinus canarius**..	46.5	5.6	53.2	55.6	131.4		40	42
Night heron, *Nycticorax nycticorax.. .*	45.0	6.1	49.0	49.0	126.1		39	39
Goose, *Anser domesticus*	39 5	4.0	21.0	80 0	123.5		17	65
" " "	42.0	5.8	27.4	65.5	116.7		24	56
" " "	48.8	6.2	23.4	71.5	112.2		21	64
" " "	32.1	4.9	19.0	67.2	108.5		18	62
Demoiselle crane, *Anthropoides virgo* . ..	41.2	4.9	28.7	66 2	117.8		24	56
Parrakeet, *psittacula spengeli**..	53.1	8.3	26.4	77.3	115.3		23	67
Duck, *Anas boscas* .	40 2	5.7	25.9	73.9	115.1		22	64
" "	48.7	5.1	19.2	63.6	111.7		17	57
Macaw, *Ara macao*	45.9	6.4	22.6	76.3	113.1		20	68
Stork, *Ciconia alba*	44.9	5.6	26.5	63.5	111.2		24	57
Black swan, *Cygnus atratus.*	42.0	3.9	21.2	67.8	97.8		22	69
Chicken, *Gallus bankivus domesticus.. . ..*	23.2	2.4	10 5	68.1	95.2		11	71
" "	31.4	3.6	11.0	67.0	90.2		12	74
" "	36.7	5.0	11.9	61.2	90.0		13	68
" "	42.7	3.1	9.2		86.5		11	

* Pooled samples of bloods from six canaries and four parrakeets.

P in the cells varied between 90 and 135 mg. per 100 cc.   None contained phosphoglycerate, and all contained large amounts of

phytic acid. The concentration of phytic acid P varied between 49 and 87 mg. per 100 cc. of cells, accounting for from 40 to 75 per cent of the organic acid-soluble P. In eight of these bloods, the adenosine triphosphate accounted for from 20 to 30 per cent of the organic acid-soluble P. The highest concentrations of adenosine triphosphate were found in the blood cells of the canaries and the night heron, and the lowest in the blood cells of the chicken and turkey. According to Kerr (3) the bloods of the guinea-hen and partridge also should conform to the general picture of the avian bloods.

Reptiles (Table III)—Turtle blood contained around 55 mg. of organic acid-soluble P per 100 cc. of cells, and of this approximately 30 per cent was adenosine triphosphate and 40 per cent was phytic acid. None of the other bloods of reptiles, amphibia, or fish contained phytic acid. In the blood cells of the three snakes and of the Gila monster the concentration of organic acid-soluble P was high, with a high proportion of adenosine triphosphate. Samples of blood from three alligators were analyzed. Two of these were approximately 60 cm. in length, while the third was only 15 cm. in length. The concentration of organic acid-soluble P in the blood cells of the two larger alligators was definitely lower than in the cells of the third (presumably younger), but the concentration of adenosine triphosphate was about the same in the cells of all three.

The concentrations of organic acid-soluble P in the turtle blood cells are somewhat higher than McCay found (4).

Amphibia (Table III)—Of the amphibian bloods studied, the bullfrog blood cells had the lowest concentration of organic acid-soluble P, 44 mg. per 100 cc., 40 per cent of this being adenosine triphosphate and the rest unidentified. A pooled sample of bloods from green frogs had a similar composition. The erythrocytes of the *Amphiuma* are especially interesting because of their tremendous size, the largest known. In these cells the concentration of organic acid-soluble P was approximately 80 mg. per 100 cc., with a relative proportion of adenosine triphosphate similar to that of frog blood cells. The one sample of *Necturus* blood had a high concentration of organic acid-soluble P, of which a high proportion appeared to be adenosine triphosphate.

Fish (Table III)—One pooled sample of blood from the un-

Table III

Distribution of Acid-Soluble P in Bloods of Different Species of Reptiles, Amphibia, and Fish

Species	Whole blood		Cells			Proportions of acid-soluble P in	
	Volume of packed cells	Inorganic P	Adenosine triphosphate P	Phytic acid P	Organic acid-soluble P	Adenosine triphosphate	Phytic acid
	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	per cent
Reptiles							
Snapping turtle, *Chelydra serpentina*	25 6	3.1	18 4		65.0	28	
" " " "	19 3	2 4	16 7		55 9	30	
" " " ..	18 3	3 1	19 4	27 6	58 3	33	47
" " " "	32 3	6 1	17.8	24.0	57.5	31	42
" " " "	24 8	3 7	18 4	22.2	55 9	33	40
Moccasin, *Agkistrodon piscivorus*	20 2	7 0	121 3	0	165 3	73	
Water-snake, *Natrix taxispilota*	28 7	7 0	99 4	0	127 6	78	
King-snake, *Lampropeltis getulus*	18.0	5.9	96 3	0	118 2	81	
Gila monster, *Heloderma suspectum*	26.5	7.6	90 0	0	122 8	73	
Alligator, *Alligator mississipiensis*							
Length 60 cm.	15.0	2.5	8 2	0	13.9	59	
" 60 "	18.9	3 3	9.8	0	13 8	71	
" 15 "	17.5	3 2	8.7	0	21.7	40	
Amphibia							
Mud-puppy, *Necturus maculatus*	18.8	5.3	65 6	0	96.8	68	
Congo-eel, *Amphiuma tridactylum*	30.5	6.1	30 2	0	81.3	37	
" " "	23.1	5.1	27 5	0	72.6	38	
Green frog, *Rana clamata**	27.0	8 1	21.8	0	56.3	39	
Bullfrog, *Rana catesbiana*	30.6	5.4	20.9	0	45.3	46	
" " "	35.8	4.2	17.6	0	41.3	43	
" " "	25.9	5.6	19.6	0	45.5	43	
Fish							
Catfish*	37.3	7.8	31.4	0	57.4	55	
" bullhead, *Amiurus melas**	34.1	13.9	45.6	0	65.0	70	
" " " "	32.6	16.7	40.2	0	57.2	70	
Black bass, *Micropterus dolomieu**	41.2	16.9	24 6	0	39.7	62	
" " " "	43.4	17.2	19.0	0	32.7	58	

* Pooled samples of bloods from fifteen green frogs, three catfish (unidentified species), two bullhead catfish, and two black bass.

identified species of catfish listed first of the fish in Table III was examined in the fall of 1939. The following spring the bloods of bullheads, a species of catfish, and of bass, were examined. In each case the bloods of two or three fish were pooled for analysis. In the first sample, some organic acid-soluble P was found to be present in the plasma (2 mg. per 100 cc.) in agreement with the findings of McCay (4). However, in the plasma of the other samples examined later no organic acid-soluble P could be detected. The concentrations of organic acid-soluble P in the blood cells of the catfish were higher than those in the cells of the bass. Both contained a high proportion of adenosine triphosphate.

<div style="text-align:center">DISCUSSION</div>

Large differences in the phosphorus content of the bloods of different mammalian species have been recognized since the classic analyses of Abderhalden (11). Subsequent investigations have led to increased knowledge of the phosphorus compounds present in blood, but the significance of differences in their concentrations in the bloods of different species is by no means clear. To date, very few data on the composition of nucleated erythrocytes have been available. The results of the studies reported here indicate an even greater diversity in the concentrations and relative distribution of the organic acid-soluble P compounds in these cells than in the non-nucleated ones of the mammalian species.

In the majority of the mammals studied the distribution of organic acid-soluble P in the blood cells was fairly uniform. In sixteen of the twenty-two species listed in Table I the concentration of organic acid-soluble P in the blood cells was rather high, with a considerable portion accounted for as diphosphoglycerate. The four species found to have a low concentration of organic acid-soluble P in the blood cells belong to a single order, the ungulates. Since it appears that a high concentration of organic acid-soluble P, with a substantial amount of diphosphoglycerate, is characteristic of the blood cells of species phylogenetically older (as the opossum) as well as those phylogenetically younger, it is possible that the low concentration of phosphorus found in the blood cells of some of the ungulates, and perhaps of other orders, may be a secondary development in the course of their evolution. According to Kerr and Daoud (2) the hyena,

a carnivorous animal, also has a low concentration of organic acid-soluble P in the blood cells.

The distribution of phosphorus in the bloods of the different avian species studied appears to be more uniform than that found among different species of the other classes studied. It is of interest to note that the different species of birds are known also to show relatively few differences in anatomical structure when compared with the structural diversity found among the members of other classes of animals.

The data on reptiles, limited to six species, indicate a great diversity in the distribution of P in the bloods of this class. The three snakes and one lizard (Gila monster) belonging to the same order, Squamata, had remarkably similar distributions of P in their blood cells, with a high concentration of adenosine triphosphate. The alligator blood cells had a lower absolute concentration of organic acid-soluble P, but a high proportion of this also was adenosine triphosphate. The turtle blood cells, differing markedly from those of the other reptiles, showed close similarities in composition to bird blood cells. The turtle blood cells contained phytic acid and, as will be reported later, the turtle blood also had a distribution of phosphatase and phytase similar to that found in bird bloods. If such relationships have evolutionary significance they suggest a very old origin of phytic acid, probably among the most primitive reptiles. A clearer understanding of the occurrence of phytic acid in turtle and bird bloods should be obtained from further studies of the lower vertebrates.

Of the amphibian species studied, the Anura (frogs) and the Urodela (*Amphiuma, Necturus*) differed considerably. The *Necturus* had a higher concentration of organic acid-soluble P in the blood cells, resembling somewhat the phosphorus distribution in reptilian bloods. In the frog blood, 50 to 60 per cent of the organic acid-soluble P remained unidentified.

The data on fish are limited to two closely related species of teleosts, but the differences found between them suggest that further studies of this large and diversified class should be of considerable interest.

Of the several recognized and measurable fractions of organic acid-soluble P, adenosine triphosphate appears to be present in the blood cells of all species, just as it has been found in all tissue cells.

This substance seems to play an essential rôle in various metabolic activities. In addition to its recognized well defined function in glycolysis, there is increasing evidence that it is important in cell respiration. In mammalian erythrocytes, adenosine triphosphate is continuously resynthesized during glycolysis. In nucleated erythrocytes, on the other hand, Engelhardt (12) has found that adenosine triphosphate is preserved only if respiration can take place. Such findings suggest that adenosine triphosphate may participate in both aerobic and anaerobic metabolism of the erythrocytes. In both nucleated and non-nucleated erythrocytes the concentration of adenosine triphosphate varies over a wide range, but the significance of this variation is not clear. A rough correlation between the concentration of adenosine triphosphate and the rate of glycolysis in mammalian red blood cells has been suggested (13).

In man and other animals whose blood cells contain large amounts of diphosphoglycerate, this substance has been found to be important in several respects: it is formed and decomposed as an intermediate product of blood glycolysis; as a non-diffusible anion in the cells it is an important factor in the ionic equilibrium of the blood; and it appears to serve various needs of the phosphorus metabolism in the body (5). Since no other tissue has been found to contain, or to form, diphosphoglycerate, its synthesis and decomposition seem to be specialized functions of the glycolytic enzyme system of the blood cells of these species. The absence of this substance in the nucleated erythrocytes of the phylogenetically older classes suggests that the ability to form diphosphoglyceric acid is an acquisition of the mammalian blood cells.

In most of the mammalian bloods adenosine triphosphate and diphosphoglycerate together accounted for from 70 to 90 per cent of the organic acid-soluble P. Other phosphoric esters are known to occur in these bloods. In the cells of human and rabbit bloods an unidentified phosphoric ester yielding a water-soluble barium salt has been found to account for from 5 to 15 per cent of the organic acid-soluble P (14). There is reason to believe that this substance also takes part in glycolysis.

The finding of phytic acid in the bloods of birds and turtles appears to be the first demonstration that this substance occurs

in any animal, although it is widely distributed in plants. According to recent experiments made in this laboratory, phytic acid apparently does not take part in the glycolysis of avian blood, but the use of radioactive P yielded evidence of a very slow rejuvenation of this compound occurring only under aerobic conditions.

Data on cell counts and the size and hemoglobin content of the erythrocytes of these different species, collected simultaneously with the studies of phosphorus distribution in the various bloods, will be reported later. It is noteworthy here, however, that no relationship was apparent between the organic P composition and the hemoglobin content or size and shape of the erythrocytes.

SUMMARY

Data are reported on the concentrations of organic acid-soluble phosphorus compounds in the blood cells of forty-six species: twenty-two mammalian, twelve avian, six reptilian, four amphibian, and two species of fish.

In most of the mammalian bloods, the concentration of organic acid-soluble P in the cells was between 50 and 100 mg. per 100 cc., with a high proportion of phosphoglycerate. In a small group (beef, sheep, goats, deer) the concentration of organic acid-soluble P in the blood cells was much lower (9 to 15 mg. per 100 cc.) with only traces of phosphorylated glyceric acid.

In all bird bloods the concentration of organic acid-soluble P in the cells was high (90 to 135 mg. per 100 cc.), with a large proportion of phytic acid P (49 to 87 mg. per 100 cc.).

A wide diversity in the distribution of acid-soluble P was found in the reptile bloods. Of these, turtle blood alone contained phytic acid. In snake blood cells the concentration of organic acid-soluble P was high, with a large proportion of adenosine triphosphate. In alligator blood cells the concentration of organic acid-soluble P was low.

Adenosine triphosphate was found in widely varying concentrations in the blood cells of all species.

Phosphoglycerate was not found in the bloods of species which normally have nucleated erythrocytes.

The possible significance of such findings in connection with evolutionary relationships among the species is discussed briefly,

as well as the possible functions of the organic acid-soluble P compounds.

BIBLIOGRAPHY

1. Kay, H. D., *Biochem. J.*, **19**, 447 (1925); *J. Physiol.*, **65**, 374 (1928).
2. Kerr, S. E., and Daoud, L., *J. Biol. Chem.*, **109**, 301 (1935).
3. Kerr, S. E., *J. Biol. Chem.*, **117**, 227 (1937).
4. McCay, C. M., *J. Biol. Chem.*, **90**, 497 (1931).
5. Guest, G. M., and Rapoport, S., *Am. J. Dis. Child.*, **58**, 1072 (1939).
6. Rapoport, S., *J. Biol. Chem.*, **135**, 403 (1940).
7. Guest, G. M., and Siler, V. E., *J. Lab. and Clin. Med.*, **19**, 757 (1934).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Rapoport, S., *Biochem. Z.*, **289**, 406 (1937).
10. Michel-Durand, E., *Bull. Soc. chim. biol.*, **20**, 413 (1938).
11. Abderhalden, E., *Z. physiol. Chem.*, **25**, 65 (1898).
12. Engelhardt, W. A., *Biochem. Z.*, **227**, 16 (1930).
13. Barrenscheen, H. K., and Vasarhelyi, B., *Biochem. Z.*, **230**, 330 (1931).
14. Morgulis, S., and Munsell, J. D., *Biochem. Z.*, **278**, 89 (1935).

SOLUBILITIES OF SOME ESTROGENS

By E. A. DOISY, Jr., MAX N. HUFFMAN, SIDNEY A. THAYER,
AND EDWARD A. DOISY

(*From the Laboratory of Biological Chemistry, St. Louis University School
of Medicine, St. Louis*)

In spite of the voluminous literature which developed following
the isolation in 1929 (1) of the first crystalline estrogen, adequate
data on solubility are still not available. The only data which the
authors have found in a search of the literature are some values
on the solubility of theelin. Butenandt and Westphal (2) reported
the solubility of theelin in water to be 2.1 mg. per liter, and de
Jongh, Kober, and Laqueur (3) gave values for chloroform, ace-
tone, and 96 per cent ethanol at their respective boiling points
and at 15°. Since more complete information was necessary for
some work in progress in this laboratory, the determination of
the solubilities of theelin in a number of common solvents was
undertaken. When the value ·of these results became apparent,
the work was extended to include all of the estrogens available
to us (α-dihydrotheelin, theelol, and equilenin).

EXPERIMENTAL

The estrogens were highly purified specimens obtained by re-
crystallization until the melting points were satisfactory. The
solvents used were purified by conventional procedures. The
saturated solutions were prepared by shaking sealed glass tubes
containing solvent and an excess of powdered solute until equilib-
rium was reached. This procedure usually required 8 to 11 days
of continuous shaking. Equilibrium at the desired temperature
was approached from both the supersaturated and the unsaturated
states; two tubes containing solvent and an excess of solute were
heated to 100° in a boiling water bath, and two similar tubes were
cooled to 0° in a water-ice bath before they were placed in the

shaking device of the thermostat. At the conclusion of the period of shaking the crystals were separated by centrifugation[1] in the sealed or, in some instances, a stoppered tube; the solution was transferred by pipette to a weighed, glass-stoppered weighing bottle. The bottle and contents were weighed, the solvent evaporated, and the bottle and crystals weighed. In those determinations in which the weight of crystals was small, the weighings were made with a Kuhlmann microbalance.

The temperatures selected for this work were 10° and 30°. The manufacturers stated that the thermoregulators would control the

TABLE I

Solubilities of Estrogens Expressed in Gm. of Solute per 100 Gm of Solvent

Solvent	Theelol or estriol		Theelin or estrone		α-Dihydrotheelin or α-estradiol		Equilenin	
	10°	30°	10°	30°	10°	30°	10°	30°
Methanol	1 66	2.02	0 33	0 63	3 03	4.1	0.665	0.982
Ethanol, 95%	1.20	1 65	0 35	0.666	2.29	3 56	0 753	1.12
Butanol .	0.567	0.738	0 33	0.60	2.61	4.15	0 500	0.943
Butyl ether .		0.0053	0 041	0.071	0.093	0.162	0 066	0 105
Benzene			0.056	0.13	0 022	0.054	0 093	0.130
Toluene		0.0034	0 052	0 099	0.015	0.0456	0.058	0 101
Acetone .	0.27	0.414	1.21	1.78	4.59	7.24	1.70	4.09
Chloroform		0.0032	0 255	0 53	0 092	0 087	0.18	0.388
Dioxane	0.63	0.97	2.11	2 84			4.69	6.35
Pyridine	31.1	33.8						

temperature to 0.1° or less but in these experiments the variation was slightly greater. The centrifugation, which required less than 10 minutes, was conducted at temperatures which varied from the temperature at which equilibrium had been established by less than 2°. Although more rigid control of the factors men-

[1] Before the adoption of centrifugation to separate the crystals, the separation by forcing the solutions through a fine, Jena sintered glass funnel was studied. Owing to adsorption of the solute on the glass, this procedure was abandoned for all solutions except those in chloroform. Since owing to the density of the chloroform the crystals would not pack in the bottom of the tube, these solutions were filtered through sintered glass filters. To minimize the effect of adsorption the first part of the filtrate was discarded.

tioned in this paragraph would have given slightly more accurate results, the data have already proved to be valuable for investigations in this department and in other laboratories.

Although all of the solvents were carefully purified, color developed before the end of the period of shaking in the tubes containing chloroform, dioxane, and pyridine. For this reason, it seems likely that the values with these solvents may be too high.

Blank spaces in the theelol column (Table I) are due to the failure to obtain satisfactory results by our procedure on account of the very low solubility of theelol in butyl ether, benzene, toluene, and chloroform.

Since petroleum ether has been extensively used in the purification of estrogens, the solubility of each of the four compounds at 30° has been determined. The boiling point range of the petroleum ether used was 89–92°. We are indebted to J. T. Van Bruggen for the following values which were obtained by a colorimetric procedure: theelol 0 71, theelin 8.9, α-dihydrotheelin 5.0, and equilenin 9.8 γ per cc.

BIBLIOGRAPHY

1. Doisy, E. A., Veler, C. D , and Thayer, S A., *Am. J. Physiol.*, **90**, 329 (1929).
2. Butenandt, A., and Westphal, U., *Z. physiol. Chem.*, **223**, 147 (1934).
3. de Jongh, S. E., Kober, S , and Laqueur, E., *Biochem. Z.*, **240**, 247 (1931).

THE UTILIZATION OF β-HYDROXYBUTYRIC ACID BY THE LACTATING MAMMARY GLAND

By J. C. SHAW and C. B. KNODT*

(*From the Department of Dairy Industry, Storrs Agricultural Experiment Station, Storrs, Connecticut*)

The arteriovenous studies of Shaw and Petersen (1, 2) have demonstrated that most of the milk fat is derived from blood fat. No consideration, however, has been given to the rôle that the intermediate substance, β-hydroxybutyric acid, may play in the secretion of milk. Accordingly a study of the acetone body metabolism of the gland was initiated to gain some further insight into the mechanism of the fat metabolism of the gland. That the utilization of ketone bodies by normal tissues is quite high has been shown by Marriott (3), Snapper and Grünbaum (4), Barnes, MacKay, Moe, and Visscher (5), and others. It appeared possible that an actively secreting gland such as the lactating mammary gland of the cow would use acetone bodies for energy purposes if not indeed for actual synthesis, although there also remained the possibility of the production of acetone bodies as the result of fat oxidation in the gland.

Methods

Arterial and mammary venous bloods were drawn as described by Shaw and Petersen (2, 6). The skin was anesthetized at the point of venipuncture with ethyl chloride to avoid undue disturbance to the animal. Arterial and mammary venous bloods were always drawn simultaneously and within a minute following the venipuncture. Concentration changes in the blood of the mammary gland were used as the basis in determining whether or not the values obtained could be expected to represent the normal metabolism of the gland. This was based on the observations

---

* Chas. M. Cox Company Fellow.

(6) that any disturbance to the animal during the drawing of the blood samples invariably resulted in significant blood concentration changes in the gland. Coincidentally the arteriovenous differences of the less freely diffusible blood precursors of milk were extremely variable, whereas the arteriovenous differences of the more freely diffusible substances were not so greatly affected. These phenomena were not observed in the completely undisturbed animal.

Blood ketones were determined by the method of Barnes and Wick (7). These analyses were always conducted immediately following the drawing of the blood samples. Hemoglobin determinations for the calculation of blood concentration changes were made by the Evelyn-Malloy method (8).

Results

A few initial experiments demonstrated that ketone bodies were used in substantial quantities by the lactating gland. A number of experiments were then conducted on both lactating and non-lactating cows in which the total ketones and the fraction, acetone and acetoacetic acid, were determined. The data presented in Table I show that there is a consistent utilization of acetone bodies by the active mammary gland. The fractionations disclose that this utilization is confined to β-hydroxybutyric acid. In the sixteen experiments on lactating cows there was a mean utilization of 2.49 ± 0.325 mg. per cent of β-hydroxybutyric acid,[1] a value which is statistically reliable as shown by the t test (9). Six experiments summarized in Table I demonstrate that the mammary gland of the non-lactating cow does not use a measurable quantity of blood acetone bodies, the mean arteriovenous difference in mg. per cent of β-hydroxybutyric acid being $+0.025 \pm 0.145$.

In eight of the experiments reported herein on lactating cows the animals were slightly agitated during the drawing of the blood samples. It will be observed that blood concentration changes occurred in the mammary gland in every case in this particular group. These changes in blood volume did not occur in the completely undisturbed group. The utilization of β-hydroxybutyric acid by the gland of the slightly agitated animal

---

[1] All such values represent standard errors throughout the present paper.

Table I

Ketone Bodies in Simultaneously Drawn Arterial and Mammary Venous Bloods of Lactating and Non-Lactating Cows

Experiment No.	Mammary blood concentration change	Acetoacetic acid + acetone (as acetone)			t	Total acetone bodies				t
						Arterial (as acetone)	Venous (as acetone)	Difference		
		Arterial	Venous	Difference				As acetone	As β-hydroxybutyric acid	
	per cent	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	mg. per cent	
				Lactating, not agitated						
1	0.00	0.63	0 64	+0.01		3.09	1 93	−1 16	−2.39	
2	0.00	0.97	1.02	+0 05		4.89	3 32	−1 57	−3.24	
3	0 00	0.99	1 42	+0.43		3.57	3 53	−0.04	−0.09	
4	0.00					4.20	2 39	−1.81	−3.74	
5	0.00	0.23	1.44	+1.21		2.22	1.53	−0.69	−1.43	
6	0 00	1.51	1 79	+0 28		3.19	2 39	−0 80	−1.65	
7	0.00	2.72	2.85	+0.13		4.95	3.55	−1 40	−2.89	
8	0.00	0.76	0.85	+0.09		3.04	2 05	−0.99	−2.04	
Average		1 12	1.43	+0 314 ±0.158*	1 987	3 64	2 59	−1 058	−2.184 ±0.408	5.353
				Lactating, slightly agitated						
9	+2.75	0.30	0.21	−0.09		2 82	1.96	−0 86	−1.78	
10	+0.26	2.66	2 76	+0.10		9 62	7.47	−2 15	−4.44	
11	−1.31	2.06	1.60	−0.46		3 32	1 66	−1 66	−3.43	
12	+0.68	0.79	1.03	+0 24		3.67	2.08	−1 59	−3 28	
13	−0 87	0.02	0.44	+0.42		2 39	2.18	−0.21	−0.43	
14	−1.01	0.24	0.28	+0.04		2 52	1 73	−0.79	−1.64	
15	−2.25	1 12	1 29	+0.17		3.22	1 87	−1.35	−2 79	
16	−2 91	1 07	1.07	0 00		3 31	1.09	−2 22	−4 59	
Average		1.03	1 09	+0 053 ±0 092	0.576	3 86	2.51	−1.354	−2.798 ±0.509	5.497
Average of all lactating cows		1.07	1 25	+0.174 ±0 092	1 891	3.75	2.55	−1.206	−2.491 ±0.325	7 665
				Non-lactating						
17	0 00					1.36	1 54	+0.18	+0.37	
18	−0.91	0.89	0.93	+0.04		2.73	2 73	0 00	0.00	
19	−0.58	2.30	2 35	+0.05		4.86	4 67	−0 19	−0.39	
20	+0.65	3 55	3 39	−0.16		5.07	5 06	−0 01	−0.02	
21	−2.15	0 49	0.43	−0.06		2 65	2 50	−0 15	−0 31	
22	−0 73					2 43	2 67	+0 24	+0 50	
Average		1.81	1.77	−0.033 ±0.049	0 673	3.18	3.20	+0.012	+0 025 ±0.145	0.172

* All such values are standard errors.

did not differ materially from that of the undisturbed animal as shown by the statistical treatment of these data. In the case of the fraction, acetone and acetoacetic acid, the effect of excitation was more noticeable upon the arteriovenous difference. In the undisturbed group there was a mean concentration in the venous bloods of 0.314 ± 0.158 mg. per cent, whereas the venous concentration of the fraction was negligible in the disturbed group. The t test for the significance of the mean arteriovenous differences in the undisturbed group suggests that acetoacetic acid, or acetone, or both are formed in small amounts in the active mammary gland, probably from β-hydroxybutyric acid.

<div align="center">DISCUSSION</div>

In view of the preceding we can conclude that β-hydroxybutyric acid is used by the lactating gland in the elaboration of milk. This utilization is not materially affected by mild disturbance of the animal, being similar to glucose and amino acids in this respect. The effects of excitation, herein noted, upon blood concentration changes in the gland confirm the earlier observations of Shaw and Petersen (6).

It is apparent that the lactating cow must mobilize considerably more ketone bodies than the non-lactating cow. Ketosis in dairy cattle generally occurs shortly after parturition and usually in the higher producing cows. According to our arteriovenous difference figures, on the basis of 500 volumes of blood per unit of volume of milk, a cow producing 20 kilos of milk per day must mobilize a total of 237.0 gm. of β-hydroxybutyric acid per day for milk secretion. It is conceivable that the onset of ketosis following parturition may be the result of a temporary imbalance in the mobilization of ketone bodies following the sudden initiation of milk secretion.

Extensive data by Shaw and Petersen (2) show an uptake by the active mammary gland of 9.0 mg. per cent of plasma fat and 0.29 mg. per cent of plasma calcium. On the basis of the quantity of calcium in the milk these data indicate a ratio of volume of blood plasma traversing the gland to the amount of milk secreted of approximately 400:1. Calculated on the whole blood basis (30 per cent erythrocytes) this ratio becomes 520:1. The quantity of short chain fatty acids in the milk (C_{14} and lower) is roughly 1200

mg. per cent. The ratio of the arteriovenous difference of 2.49 mg. per cent of β-hydroxybutyric acid to the quantity of short chain fatty acids in the milk is approximately 1:480. The quantity of β-hydroxybutyric acid used by the active gland is therefore sufficient to account for the short chain fatty acids of milk which are not found in significant quantities in blood fat. Such a conversion would explain the high respiratory quotient of the lactating mammary gland reported by Graham et al. (10) and confirmed by Shaw and Petersen (11) and Reineke (12).

However, since β-hydroxybutyric acid is oxidized so readily by the various body tissues, it is quite possible that it is used as a source of energy by the actively secreting gland. The resultant respiratory quotient of the gland would tend to be low, however, and considerable fat synthesis from carbohydrate material would have to take place to account for the high respiratory quotient of the active gland. Since most of the milk fat is derived from blood fat (6), a synthesis of fatty acids other than the short chain fatty acids does not seem to be indicated. Likewise the extensive data of Shaw, Boyd, and Petersen (13) on the utilization of glucose and lactic acid by the gland for lactose synthesis do not indicate that there is any considerable amount of carbohydrate material available for such synthesis. Assuming that 1.0 mg. of β-hydroxybutyric acid requires 0.96 cc. of O_2 for complete combustion, approximately 2 cc. of oxygen would be used per 100 cc. of blood traversing the gland completely to oxidize the β-hydroxybutyric acid taken up by the secreting gland. A large number of blood analyses by Petersen and Shaw[2] show a mean utilization of oxygen by the active gland of the undisturbed cow of 4.8 per cent. If the β-hydroxybutyric acid is completely oxidized for energy purposes, 42 per cent of the total oxygen consumption of the secreting gland can be accounted for by ketone body utilization.

SUMMARY

β-Hydroxybutyric acid is used in substantial quantities by the lactating mammary gland of the cow. The non-lactating gland does not use a measurable quantity of ketone bodies.

Evidence is presented to show that β-hydroxybutyric acid may

[2] Petersen, W. E., and Shaw, J. C., to be published.

be the precursor of the short chain fatty acids of milk. If, however, it is all used for energy purposes, approximately 42 per cent of the total oxygen consumption of the secreting gland would be due to β-hydroxybutyrate burning.

BIBLIOGRAPHY

1. Shaw, J. C., and Petersen, W. E., *Am. J. Physiol.*, **123**, 183 (1938).
2. Shaw, J. C., and Petersen, W. E., *J. Dairy Sc.*, **23**, 1045 (1940).
3. Marriott, W. M., *J. Biol. Chem.*, **18**, 241 (1914).
4. Snapper, I., and Grunbaum, A., *Biochem. Z.*, **201**, 464 (1928).
5. Barnes, R. H., MacKay, E. M., Moe, G. K., and Visscher, M. B., *Am. J. Physiol.*, **123**, 272 (1938).
6. Shaw, J. C., and Petersen, W. E., *Proc. Soc. Exp. Biol. and Med.*, **42**, 520 (1939).
7. Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, **131**, 413 (1939).
8. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, **126**, 655 (1938).
9. Fisher, R. A., Statistical methods for research workers, Edinburgh, 5th edition (1934).
10. Graham, W. R., Jr., Houchin, O. B., Peterson, V. E., and Turner, C. W., *Am. J. Physiol.*, **122**, 150 (1938).
11. Shaw, J. C., and Petersen, W. E., *J. Dairy Sc.*, **23**, 538 (1940).
12. Reineke, E. P., *J. Dairy Sc.*, **23**, 543 (1940).
13. Shaw, J. C., Boyd, W. L., and Petersen, W. E., *Proc. Soc. Exp. Biol. and Med.*, **38**, 579 (1938).

THE CATALYTIC EFFECT OF ACTIVE CRYSTALLINE PAPAIN ON THE DENATURATION OF THYROGLOBULIN*

By HAROLD P. LUNDGREN

(*From the Department of Chemistry, University of Wisconsin, Madison*)

It has been assumed that denaturation occurs preliminary to enzymatic hydrolytic fission in some proteins. The basis of this supposition is the generally accepted observation that these proteins are less readily attacked by proteolytic enzymes in their native state than when they are denatured (1–3). In the case of such readily digestible proteins as gelatin, prolamin, or casein, Linderström-Lang states that there is hardly any doubt that fission of peptide bonds is the initial and sole reaction, but in the case of the slowly digestible "crystalline or globular proteins" he believes that a secondary structure in the native protein blocks the peptide bonds to direct enzyme action. Such a blocking is thought to have its origin in steric hindrances or in the presence of chemical structures into which peptide bonds have themselves entered.

To account for the difference between the action of proteolytic enzymes on the native and denatured forms of the slowly digestible proteins and in the absence of the proof of a direct action of enzymes on the native proteins Linderstrom-Lang proposes that the process involves reversible denaturation. He assumes that native proteins as such are not attacked by the enzyme, but the change occurs as a result of an equilibrium between the native, N, and denatured, D, forms. Then, if the proteolytic enzyme promotes the removal of the denatured form by hydrolysis, the native form shifts to D to maintain the equilibrium as follows:
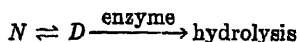
$$N \rightleftharpoons D \xrightarrow{\text{enzyme}} \text{hydrolysis}$$

In this investigation the rôle which a proteolytic enzyme plays in denaturation prior to enzymatic hydrolysis has been determined through use of newer physicochemical methods. In this paper there are reported the results of a study on the effect of crystalline active papain on a system in which the changes occurring in denaturation are already known and can be followed under rigid experimental control. Highly purified hog thyroglobulin offered the ideal substrate.

In previous studies on the stability characteristics of thyroglobulin (4, 5) it was found by ultracentrifugal analysis that given appropriate conditions it is possible to detect the formation from the native form of the protein of an unstable more slowly sedimenting protein, called for convenience α-protein. An equilibrium is finally established. This unstable form was found to behave as an intermediate between the native protein on the one hand and the denatured form on the other. It appears to involve an unfolding of the regular structure in the native protein molecule preliminary to a change into the new structure of the denatured form (5). A similar mechanism has been found to hold in the case of several other proteins so far investigated in this laboratory. The effect of the addition of salt is to reverse the process with formation of native protein. This accounts for the fact that the α form is not observed in electrolyte concentrations ordinarily used in the sedimentation analysis of proteins. Conditions favoring denaturation such as heat treatment or addition of increasing amounts of sodium salicylate to the solution cause the disappearance of α-protein with the simultaneous formation of denatured protein. The denatured form sediments at the same rate as the original native protein and in the presence of salt the denatured protein appears to be heterogeneous. Aside from this, the denatured form can be distinguished from the native protein by differences in solubility and electrophoretic mobility.

After denaturation caused by the heating of the salt-free native thyroglobulin solution, the protein possesses different solubility, because it precipitates in borax-succinic acid buffers at pH 5.2, ionic strength = 0.1. The native protein is soluble under these conditions. On standing in this solution, especially at elevated temperatures, the native form changes to the denatured form and precipitates. On the other hand if the cooled heat-denatured

protein is allowed to stand before addition of the buffer, the precipitability is decreased as before. On reheating, followed by cooling, the precipitability again decreases on standing. These experiments point to the reversibility of denaturation in this protein under these conditions.

Effect of Active Papain on Denaturation in the System, $N \rightharpoonup \alpha \rightharpoonup D$, under Conditions Favoring Retention of Denatured Form in Solution: Ultracentrifugal Analysis

Salt-free solutions of thyroglobulin, with a total protein concentration of 2.0 per cent and containing the equilibrium mixture,
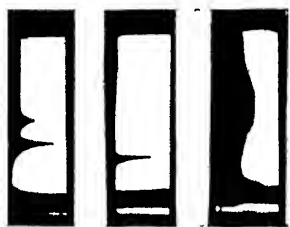
FIG. 1 Ultracentrifugal diagrams obtained by using the Philpot schlieren optical system, showing the effect of active papain on α-thyroglobulin. The left-hand diagram represents the results of an experiment performed before incubation but immediately after mixture with papain at 40°. Native protein is shown below and α-protein above. (Thyroglobulin sediments to the bottom of the centrifuge cell before papain comes into the optical field, therefore the papain peak is not to be observed in these pictures. Thyroglobulin $s_{20} = 19\ 2 \times 10^{-13}$, papain $s_{20} = 2\ 7 \times 10^{-13}$) The center diagram represents the results of an experiment after incubation with papain for 3 hours at 40° No α-protein is present The right-hand diagram represents the results of an experiment after incubation with papain for 12 hours at 40° Hydrolytic fission is shown Thyroglobulin concentration $= 2\ 3 \times 10^{-5}$ mole per liter, papain concentration $= 8\ 8 \times 10^{-6}$ mole per liter, NaCN concentration $= 8\ 0 \times 10^{-4}$ mole per liter; pH $= 5\ 6$.

$N \rightleftharpoons \alpha$, established at pH 5.8, were incubated at temperatures which previously had been found to cause the slow disappearance of α-protein into the denatured form. At intervals samples were removed and subjected to ultracentrifugal analysis in a Svedberg oil turbine ultracentrifuge With the Philpot modification of the schlieren optical system it was found that the presence of active papain catalyzed the disappearance of α-protein (Fig. 1).

Control experiments proved that this effect was due to the active enzyme present and not to the presence of electrolytes, including HCN. Furthermore, papain preparations which had been denatured by heat treatments in a water bath at 100° were not

TABLE I

Effect of Papain on α-Thyroglobulin As Shown by Ultracentrifugal Analysis

Thyro-globulin	Papain	NaCN	pH	t	Time of standing	Relative amount of α-protein to normal native protein present
moles per l. × 10⁵	moles per l × 10⁶	moles per l. × 10⁴		°C	hrs	
5 8			5 6	25		About 50% normal sedimenting component
5 8			5 6	40	28	About same relative amount
3 7			5 6	25		About 50% normal component
3 7			5 6	40	47	Slightly less than native sedimenting component
2 3	8 8 (Active)	8 0	5 8	25		About 25% normal sedimenting component
2 3	8 8 "	8 0	5 8	26 6	12	Very little, less than 5% normal sedimenting component
2 3	8 8 "	8.0	5 8	26.6	72	All gone
2 3	8 8 "	5.00	5 65	25		25% normal sedimenting component
2 3	8.8 "	8 0	5 65	30	22	None
2 3	2 2 "	4 0	5 6	25		25% normal sedimenting component
2 3	2 2 "	4.0	5 6	30	12	None
2 3	2 2 "	4.0	5 6	30	36	"
2 3	8 8 (Denatured, 100°, 10 min)	8 0	5 65	25		25% normal sedimenting component
2 3	" "	8.0	5 65	30	14	" "

effective. With native protein at the start and with no α form present, incubation at temperatures between 30–50°, with or without active papain, caused no appearance of α-protein as shown by centrifugal analysis. This is explained on the basis of the relative reaction rates, by assuming that the α form changes to

the denatured form in papain catalysis. Under these conditions the rate of change of α-protein to the denatured, D, form is greater than the change of native protein to the α condition.

When the temperature is below 30°, the rate of the reaction $\alpha \rightarrow D$ becomes slower than that of the reaction $N \rightarrow \alpha$, since α-protein accumulates in the solution. Thus the change $\alpha \rightarrow D$ appears to be the rate-determining step in denaturation.

The time intervals involved in these experiments were in no case long enough to cause significant hydrolytic fission, since no lighter material was detected in the sedimentation field. The results of a typical sequence of experiments are given in Table I.

Although the catalytic disappearance of α-protein in the presence of papain is assumed to involve the simultaneous formation of the soluble denatured form of the protein (as had previously been shown to occur in the absence of enzyme) confirmation was necessary, since the denatured protein cannot be distinguished from the normal native protein by centrifugal analysis alone. The system was studied further by using the methods of differential solubility and electrophoresis.

Effect of Active Papain on Denaturation of Thyroglobulin under Conditions Favoring Precipitation of Denatured Protein As Rapidly As It Is Formed: Kinetics of Denaturation

To render the differential solubility method more adaptable for the kinetic investigation of the system, it was decided to follow the precipitation in borax-succinic acid buffers by means of changes in light absorption as detected by using a photoelectric colorimeter. Preliminary experiments showed that for all practical purposes the light absorption of the suspension of the precipitated denatured, D, form (especially when the initial concentration of D was not too high) varied with concentration according to Beer's law.

An apparatus was designed for studying the light absorption in thyroglobulin systems at various temperatures. This consisted of a General Electric photoelectric cell (No. 88 × 565), a Rawson milliammeter, a constant light source, and a constant temperature bath with arrangements for passing monochromatic light through the solutions and onto the photoelectric cell.

A standardized experimental procedure was developed. The

water bath was regulated to the desired temperature ±0.05°. To insure complete temperature equilibrium the buffer was at-tempered in the experimental tube before each experiment. With 10 cc. of buffer attemperated at 46.3°, the addition of 0.5 cc. of 3.5 per cent thyroglobulin at room temperature caused no change in temperature of any practical significance. In most experiments not more than 0.2 cc. of 3.5 per cent thyroglobulin and not more than 0.04 cc. of 0.68 per cent papain solution were added for any experiment. Readings were taken at appropriate time intervals with intermittent stirring. The light intensity, regulated at scale reading 100 on the milliammeter with 10 cc. of buffer alone, was checked at the end of the reaction or, if the reaction was very slow, from time to time with a matched tube containing 10 cc. of buffer solution. In all experiments reported here, there was used a borax-succinic acid buffer at pH 5.2, with ionic strength = 0.1.

In a typical experiment, a solution of 3.5 per cent thyroglobulin was denatured by heating in a boiling water bath for 10 minutes. The solution was cooled to room temperature and 0.2 cc. aliquots were added immediately to 10 cc. of buffer attemperated at 46.3°. The precipitation curves were reproducible to within 2 per cent. There is shown in Fig. 2 the curve for precipitation of denatured protein under these conditions. Here the ordinate represents the concentration of denatured protein precipitated, and is equal to $\log (I_0/I)/K$. In this equation I_0 is the original light intensity as registered by the milliammeter, I is the intensity of light passing through the suspension at time t, and K is the extinction coefficient.

When the solutions of denatured protein were allowed to stand, an appreciable and regular decrease in the over-all rate of precipi-tation was observed. When the material was reheated, cooled rapidly, and analyzed, the precipitation rate was as fast as before. This again indicates that on standing reversible denaturation occurs in the cooled solution.

In the presence of active papain the precipitation of denatured protein was not affected. However, solutions which had stood for a period of time and which precipitated somewhat more slowly than the freshly prepared denatured form now precipitated as fast as the freshly denatured protein, showing that papain cata-lyzes the denaturation of the reversed protein.

With native protein as the starting material, with or without active papain, the rate of precipitation follows the general form of the curves shown in Fig. 3. From kinetic considerations, it is possible to show the effect of papain on the step involving the denaturation of the protein. It has been well established that the denaturation reaction follows the first order reaction law. Hence the rate of formation of denatured protein is expressed as follows·

$$(1) \qquad (dD/dt)_f = N_0 e^{-k_1 t}$$
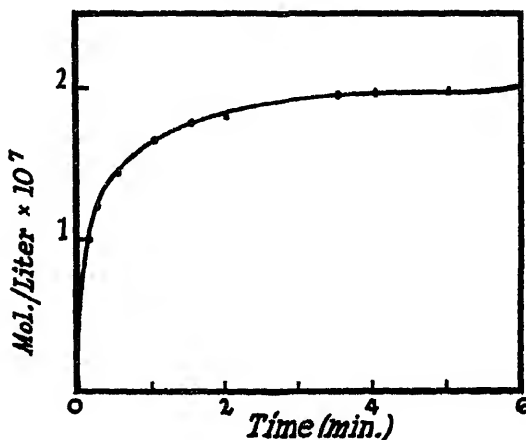
FIG. 2. Precipitation of denatured thyroglobulin, with the denatured form in succinic acid-borax buffer as starting material, pH 5.2; $\mu = 0.1$; $t = 46.3°$.

In this equation N_0 is the original concentration of native protein and k_1 is the first order reaction velocity constant. This equation expresses the rate of the slowest reaction governing the change, $N \to \alpha \to D$.

When precipitation occurs, the rate of change of concentration of denatured soluble protein is equal to the rate of formation of denatured protein minus its rate of precipitation, or,

$$(2) \qquad \left(\frac{dD}{dt}\right)_s = \left(\frac{dD}{dt}\right)_f - \left(\frac{dD}{dt}\right)_p$$

But when the rate of formation of denatured protein is equal to the rate of precipitation, the system is in the steady state and

$$(3) \qquad \left(\frac{dD}{dt}\right)_s = 0$$

so that

$$(4) \qquad \left(\frac{dD}{dt}\right)_p = \left(\frac{dD}{dt}\right)_f = N_0 e^{-k_1 t}$$
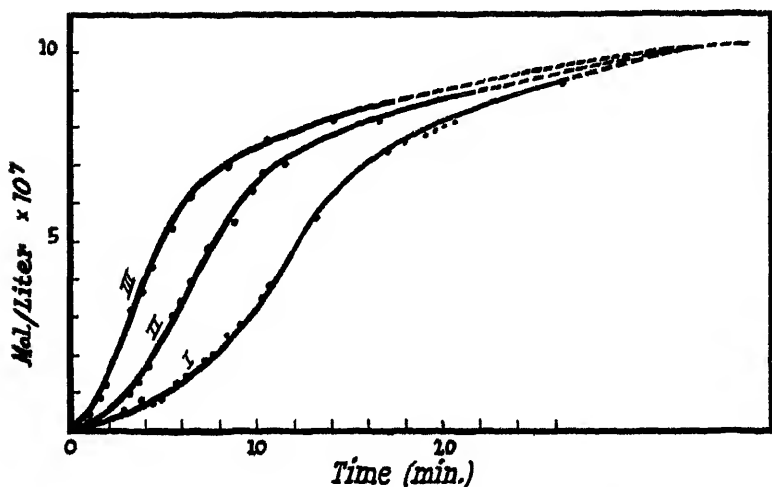
Fɪɢ. 3. Precipitation of denatured thyroglobulin, with the native form in succinic acid-borax buffer as starting material; pH 5.2; $\mu = 0.1$; $t = 46.3°$. Curve I represents the control ($N \rightarrow D \rightarrow$ precipitate); Curve II, the control $+ 4.5 \times 10^{-9}$ mole of active papain; Curve III, the control $+ 9.0 \times 10^{-9}$ mole of active papain. Papain activity units by the milk clotting method per mg. of protein nitrogen $= 21.3$.

Therefore if the steady state can be recognized, it is possible to determine the reaction velocity constant, k_1, for the first order denaturation reaction in this system. By plotting the logarithm of the concentration of the precipitating denatured protein against time, the steady state should exist in the region where the curves are linear. This region was found to occur early in the reaction. The k_1 values corresponding to the slopes of the straight lines are tabulated in the last column of Table II. With the k_1 values for reactions at two temperatures and with the van't Hoff-Arrhenius

equation, a value of about 100,000 calories was calculated for the critical thermal increment. Since this value is characteristic of the denaturation reaction in protein systems, the validity of the foregoing interpretation is confirmed. Not only is the reaction constant, k_1, for the denaturation reaction increased by the enzyme as shown by Table II but as the rate is increased by the enzyme

TABLE II

Kinetics of Denaturation of Thyroglobulin in Succinic Acid-Borax Buffers, pH 5.2, $\mu = 0.1$

Thyro-globulin	Papain	NaCN	t	$\dfrac{\Delta \log_e C}{\Delta t} = k_1$
moles per l. $\times 10^7$	*moles per l. $\times 10^9$*	*moles per l. $\times 10^5$*	*°C.*	
2.0			46.3	0.28
2.0	4.5 (Active)	8.0	46.3	0.67
2.0	9.0 "	8 0	46.3	2.50
2.0	9.0 (Heat-denatured, 100°, 10 min.)	8.0	46.3	0.384
2.0		8.0	46.3	0.29
2.0	9.0 (Inactive, oxidized)		46.3	0.36
2.0			46.3	0.29
2.0			50.4	2.5

TABLE III

Electrophoretic Mobility of Denatured Thyroglobulin Catalyzed by Papain

3.02 per cent thyroglobulin (pH 5.8, electrolyte-free), 0.07 per cent active papain, and 0.02 M NaCN were incubated at 40° for 3 hours and diluted 1:15 with succinic acid-borax buffer at 0°; pH, 5.2; $\mu = 0.1$.

Procedure	Average mobility of boundary		Average mobility of thyroglobulin, $\mu = 0.02$, pH 5.2	
	Forward	Backward	Native	Denatured
Dialyzed 10 hrs. at 5° .	5.14	4.95	2.0	5.5
No dialysis run immediately .	5.10	4.91		

the S-shaped curves of Fig. 3 approach the characteristic shape of the curve for the precipitation of denatured thyroglobulin which is shown in Fig. 2. The k_1 values for the effect of inactive and denatured papain and for the presence of cyanide alone (Table II) prove that only the active papain catalyzes the denaturation of thyroglobulin.

Electrophoretic Mobility of Papain-Catalyzed Denatured Thyroglobulin

Evidence that α-protein changes to the denatured form on heat treatment in the absence of enzyme was presented in another paper (5). By the addition of buffer to the solution and analysis in a Tiselius electrophoresis apparatus, mixtures of the heat-treated protein and native protein in equal concentration showed two boundaries of approximately equal size. These boundaries were found to have mobilities which correspond to values found earlier for native and denatured thyroglobulin by Heidelberger and Pedersen (6).

Solutions containing the equilibrium system $N \rightleftharpoons \alpha$ were incubated with active papain until they showed no α-protein on centrifugal analysis. They were then treated with buffer solution and analyzed in the Tiselius equipment. A single boundary with mobility corresponding to that of the denatured form prepared without enzyme was obtained.

Judging from the electrophoretic field, the extent of hydrolytic fission during the denaturation under these conditions appears negligible.

The results of these experiments are given in Table III.

DISCUSSION

The studies of the kinetics of precipitation and of the electrophoretic behavior in thyroglobulin systems confirm the observations made by ultracentrifugal analysis which show that active papain catalyzes the denaturation of thyroglobulin under conditions in which hydrolysis is negligible. The catalysis involves the reaction between the unstable or α form of the protein and the denatured form. Since the denaturation reaction follows the first order law, the reaction, native protein to α form, must occur rapidly under the conditions in these experiments. Accordingly, under conditions in which the native protein is stable, the enzyme would have no effect. Since the production of α-protein appears to involve an unfolding of the protein in salt-free solutions, the assumption of Linderström-Lang that structural blocking opposes enzyme action in the native protein is confirmed. In salt solution, unfolding itself is not observed on sedimentation

analysis. It has not yet been determined whether, under ordinary conditions, this formation of α-protein occurs too fast to be followed or perhaps involves a less profound change (*cf.* Annetts (7)) of the molecule.

The results confirm the assumption that denaturation is an initial reaction in proteolysis. It appears non-essential in the system under investigation whether or not the change from native protein to the denatured form is reversible.

SUMMARY

Ultracentrifugal, kinetic, and electrophoretic experiments show that active papain catalyzes the denaturation of thyroglobulin prior to hydrolytic fission of the protein. Inactive native or denatured papain does not possess this activity. An initial non-enzymatic conditioning reaction occurs before the enzymatic reaction. This involves a structural change in the native protein which presumably liberates groups to serve as points of attack for the enzyme. The results are in agreement with the Linderström-Lang theory for the mechanism of enzyme action in protein systems.

BIBLIOGRAPHY

1. Linderström-Lang, K., Hotchkiss, R. D., and Johansen, G., *Nature*, **142**, 996 (1938).
2. Linderström-Lang, K., *Proc. Roy. Soc. London, Series B*, **127**, 17 (1939); in Luck, J. M., Annual review of biochemistry, Stanford University, **8**, 45 (1939).
3. Balls, A. K., in Luck, J. M., Annual review of biochemistry, Stanford University, **9**, 44 (1940).
4. Lundgren, H. P., *Nature*, **138**, 122 (1936); **143**, 899 (1939); *J. Chem. Physic.*, **6**, 177 (1938).
5. Lundgren, H. P., and Williams, J. W., *J. Physic. Chem.*, **43**, 989 (1939).
6. Heidelberger, M., and Pedersen, K. O., *J. Gen. Physiol.*, **19**, 95 (1935).
7. Annetts, M., *Biochem. J.*, **30**, 1807 (1936).

.

A METHOD FOR THE ISOLATION OF DIPHOSPHOPYRI-DINE NUCLEOTIDE

By BERNHARD J. JANDORF

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

Several methods for the isolation of diphosphopyridine nucleotide, often referred to as DPN or cozymase, have appeared since its importance in biological oxidation systems became evident (1, 2, 3). The most recent methods (2, 4, 5), however, still suffer from the disadvantage that either they require a number of steps involving considerable loss, or large quantities of expensive chemicals are needed.

The method to be described below involves fewer operations and relatively inexpensive reagents. The yields are of the same order as those in other procedures. By adsorption of diphosphopyridine nucleotide on charcoal and elution with amyl alcohol, several of the steps involving heavy metal and acetone precipitations have been eliminated.

The adsorption of another nucleotide, adenosine triphosphate, on charcoal has been previously described (6), but this procedure has to our knowledge not been used in the preparation of nucleotides.

Myrbäck and Larsson (7) state that animal charcoal adsorbs little diphosphopyridine nucleotide in acid solution; we have found it possible, however, to adsorb it quantitatively on charcoal (norit) in weakly acid solutions. The over-all yield of adsorption and elution is usually 80 to 85 per cent.

Yeast (4, 5) or red blood cells (2) are the source of diphosphopyridine nucleotide generally employed. The former material was chosen as the source in our work.

Method

Principle—The proteins from a hot yeast extract are removed by lead. After elimination of excess lead and adjustment of pH,

the solution is treated with charcoal, the latter being eluted with an amyl alcohol-water mixture.   After concentration, the nucleotide is precipitated by cuprous chloride, the salt decomposed by H_2S, and the nucleotide isolated from the concentrated filtrate by precipitation with ethyl alcohol.

Lead Precipitation—Fresh brewers' top yeast[1] is filtered by suction on large Buchner funnels, washed once with water, and sucked dry until the cakes begin to break up.   4 kilos of the yeast cake are broken up into small particles and transferred gradually to 2 liters of approximately 0.5 N HCl with mechanical stirring.   The temperature is kept between 80–85° during this process and is maintained for a further 10 minutes.   With continued stirring, a warm solution of 480 gm. of neutral lead acetate in 800 cc. of water is added in a thin stream.   The mixture is centrifuged and the precipitate discarded.   The supernatant solution is treated with a warm solution of 180 gm. of anhydrous sodium sulfate in 700 cc. of water and centrifuged after repeated stirring.   To the resulting solution, 10 N NaOH is added, drop by drop, with vigorous stirring, until neutral to brom-thymol blue.   The resulting 4 liters of a light yellow, slightly opaque solution will be referred to as the *Pb filtrate*.

Charcoal Adsorption and Elution—To the Pb filtrate, 60 gm. of charcoal are added, and the mixture is shaken mechanically for 3 hours.   The charcoal is removed by centrifugation, washed twice with water, and transferred to a large flask (capacity 10 to 12 liters),[2] 360 cc. of amyl alcohol are added, and the total volume brought to 6 liters with water.   This mixture is shaken for 20 to 24 hours mechanically and filtered by suction, and the charcoal washed twice with 50 cc. portions of a 6 per cent amyl alcohol-water mixture.   The combined solutions are concentrated to a known volume (300 to 500 cc.) by vacuum distillation at a bath temperature of 65–70°.   The clear yellow solution is designated

[1] Made available through the generosity of the Croft Brewing Company, Boston.

[2] The ease of centrifuging the adsorbate varied with different samples of charcoal.   Although the charcoal was sometimes difficult to remove by centrifugation, the yield of diphosphopyridine nucleotide was not materially decreased.   Initial centrifugation of the charcoal adsorbate requires at least 1 hour, but subsequent centrifugations for the purpose of washing the adsorbate usually require only 20 minutes.

as the *eluate*. The diphosphopyridine nucleotide content of the eluate is determined before we proceed with the next step.

Cuprous Chloride Precipitation—The procedure followed in this step is mainly that evolved by von Euler *et al.* (4). The eluate in 100 cc. fractions is precipitated under nitrogen with 1 gm. of Cu_2Cl_2 (freshly prepared and free from cupric salts, dissolved in 10 cc. of 25 per cent KCl containing 0.1 per cent HCl) for each 50 mg. of diphosphopyridine nucleotide present. The mixture is centrifuged, and the precipitate washed twice in the centrifuge with water saturated with nitrogen. The precipitate is ground in a mortar with small amounts of water and transferred to a cylinder through which a stream of H_2S passes (final volume approximately 250 cc.). H_2S is allowed to flow for 2 hours after the last fraction of the eluate has been thus treated.

The mixture is aerated[3] and most of the cuprous sulfide removed by centrifugation. The supernatant is then filtered (filter paper, Schleicher and Schüll No. 602) to remove the remainder of the Cu_2S. The precipitates are washed once with water. The combined filtrate and washings are again treated with H_2S for 10 minutes, filtered, and again aerated.

Alcohol Precipitation—The resulting clear, faintly straw-colored solution is concentrated by vacuum distillation (bath temperature 45°) to approximately 25 cc. 1 volume of alcohol is added, and after the mixture has stood in the cold overnight, the light brown precipitate is centrifuged off and washed once with 50 per cent alcohol. To the combined solutions, a slow stream of absolute alcohol is added with constant shaking, until 90 per cent concentration of alcohol has been reached. The flocculent precipitate of diphosphopyridine nucleotide is centrifuged after standing in the cold for some hours (preferably overnight), washed twice with absolute alcohol, twice with ether, and dried *in vacuo* over H_2SO_4.

An additional small crop (at most 10 mg.) of less pure nucleotide

[3] It was found that the yield is increased by observation of the following precautions. After aeration with a moderately rapid current of air for about an hour, small test portions are centrifuged and the supernatant treated with H_2S. These tests are repeated first at 15 minute and later at 5 to 10 minute intervals until the treatment with H_2S causes a slight darkening of the color as compared with another centrifuged test portion not so treated. At this stage, aeration is stopped and the mixture worked up as described above.

can be recovered from the supernatant alcoholic solution by further concentration and reprecipitation.

<div align="center">DISCUSSION</div>

The material resulting from 4 kilos of yeast is 400 to 450 mg. of a white, hygroscopic powder, the solution of which shows no fluorescence. On drying (1.5 mm. of Hg, 60°), it loses 6 to 7 per cent of its weight. One sample (Preparation B) showed a P content of 9.32 per cent (theory, 9.35 per cent).

In accordance with the method described in the following paper (8), the activity of this preparation was compared with that of a sample of pure diphosphopyridine nucleotide prepared in Warburg's laboratory and given to us by Dr. Fritz Lipmann. The

<div align="center">TABLE I</div>

<div align="center"><i>Concentration of Diphosphopyridine Nucleotide in Steps of Isolation</i></div>

	Pb filtrate	Eluate	Final preparation
Total volume, cc. ..	3920	300	
" diphosphopyridine nucleotide, mg. ..	825	675	420
Dry weight, gm.	416 21	8.649	0.420
Diphosphopyridine nucleotide per gm. final dry weight, mg.	1.98	78.04	1000

two preparations proved to be of equal purity within the limits of accuracy of the method of estimation (*i.e.*, ±3 per cent). Through the generosity of Dr. D. E. Green, we were also able to compare our preparation with another sample of diphosphopyridine nucleotide prepared by Williamson and Green (9). This preparation is reported by various criteria to be between 63 and 68 per cent pure. By our method of determining activity, the Williamson and Green preparation proved to be 64.7 per cent as active as our Preparation B.

Seven separate preparations have been carried out by this procedure. The final products have all proved to have the same activity per unit weight as that of Preparation B.

Since the extraction of the yeast with HCl solution destroys reduced nucleotide, attempts were made to increase our yields

by adding $K_3Fe(CN)_6$ to the acid. It was found that the procedure increased the yield so little that it was abandoned.

Although many variations have been tried, it has not been possible to increase the recovery in the Cu_2Cl_2 precipitation beyond 60 per cent. Even this recovery can only be achieved by application of the precautions described above.[3]

Table I represents typical yields of diphosphopyridine nucleotide at the different steps (Preparation F). The activity of the Pb filtrate and eluate was determined by means of the manometric test. The last line in Table I shows the degree of concentration achieved at the different stages of purification.

The author wishes to thank Dr. Y. Subbarow and Dr. F. W. Klemperer for many valuable suggestions, and Dr. A. Baird Hastings for constant encouragement and guidance while this work was pursued.

SUMMARY

A simplified method for the isolation of pure diphosphopyridine nucleotide (cozymase) is described. The method involves as a new step the adsorption of diphosphopyridine nucleotide on charcoal from aqueous solution and the elution therefrom with water-amyl alcohol mixture.

BIBLIOGRAPHY

1. von Euler, H., *Ergebn. Physiol.*, **38**, 1 (1936).
2. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
3. Meyerhof, O., *Ergebn. Physiol.*, **39**, 10 (1937).
4. von Euler, H., Albers, H., and Schlenk, F., *Z. physiol. Chem.*, **240**, 113 (1936). Schlenk, F., and von Euler, H., in Zechmeister, L., Fortschritte der Chemie organischer Naturstoffe, Vienna, **1**, 99 (1938).
5. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
6 Fiske, C. H., *Proc. Nat. Acad. Sc.*, **20**, 25 (1934).
7. Myrback, K., and Larsson, H., *Z. physiol. Chem.*, **225**, 131 (1934).
8. Jandorf, B. J., Klemperer, F. W., and Hastings, A. B., *J. Biol. Chem.*, **138**, 311 (1941).
9. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).

A MANOMETRIC METHOD FOR THE DETERMINATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE

By BERNHARD J. JANDORF, FRIEDRICH W. KLEMPERER, AND A. BAIRD HASTINGS

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

Although several methods have been used for the estimation of diphosphopyridine nucleotide (cozymase), none has so far been free from certain disadvantages. In the method to be described we have attempted to avoid some of these drawbacks.

Previously described methods may be grouped in three categories:

Optical Methods—These involve the spectrophotometric measurement of the difference in extinction coefficients at 340 mμ of the material before and after reduction with hydrosulfite (1, 2, 3). Triphosphopyridine nucleotide (coenzyme II) has the same optical properties as diphosphopyridine nucleotide; by this method, therefore, the sum of the two nucleotides is determined unless reduction is carried out by means of a substrate and enzyme system specific for one of them.

Bacterial Growth Methods—Certain microorganisms, notably *Hemophilus parainfluenzae*, will grow on synthetic media only if the so called factor V is added (4). This factor can be replaced by diphosphopyridine nucleotide or triphosphopyridine nucleotide (5), a fact which has been adapted to the measurement of these two nucleotides (*e.g.* (6)). It has not been shown, however, that in biological extracts no other substance has the same growth-stimulating effect.

Manometric Methods—The methods based on the breakdown of glucose or its intermediary metabolites in the presence of certain enzymes have so far been the most promising ones, since test systems can be devised which are specifically catalyzed by di-

phosphopyridine nucleotide. A procedure has been worked out in the laboratory of von Euler (7, 8), in which the fermentation of glucose in the presence of this nucleotide, inorganic phosphate, catalytic amounts of hexose diphosphate, and a dried yeast preparation (apozymase) is followed manometrically, the rate of CO_2 evolution being proportional over a certain range to the amount of nucleotide added.

The fermentation test, whose accuracy is ± 10 per cent, suffers from the disadvantage, emphasized by the authors themselves, that the enzyme preparations are variable in their activity. Yeast from the same source sampled at different times yields apozymases of different strengths. Experimental results are, therefore, not comparable with each other, except after calibration of the apozymase preparation with known amounts of diphosphopyridine nucleotide in each determination. This variability is found to an even greater extent when apozymases from different strains of yeast are compared (7, 9). The test involves all the enzymes concerned with alcoholic fermentation, and the variability is probably due to differences in concentration of the least stable component, carboxylase. A test system which involves only a few and relatively stable enzymes would, therefore, be of advantage.

The method developed by Warburg and Christian (2), which involves the oxidation of hexose monophosphate (Robison ester) by acetaldehyde through the mediation of diphosphopyridine nucleotide and adenosine triphosphate, is the first successful attempt to measure the nucleotide in a system involving only one phase of carbohydrate breakdown. Although this method of assaying is satisfactory, the preparation of Robison ester is time-consuming and rather difficult; furthermore, the purified enzymes are not particularly stable.

Method

Principle—The present method depends upon the enzymatic conversion of hexose diphosphate into phosphoglyceric and glycerophosphoric acids, under conditions in which the rate of conversion is proportional to the amount of diphosphopyridine nucleotide present. The amount of acid produced in a given time, in the presence of bicarbonate buffer, can be measured manometrically with the Warburg apparatus.

In addition to nucleotide, buffer, and hexose diphosphate, the presence of a muscle extract as the source of enzymes, and of arsenate to limit specifically the glycolytic process, is required.

The method is adapted to the estimation of 0.5 to 5 γ of diphosphopyridine nucleotide with an accuracy of approximately $\pm 0.03 \gamma$. It has the advantage that the necessary components of the system are fewer in number, easily available, quite reproducible, and stable for at least 4 months.

The high blank reaction (*i.e.*, glycolysis in the presence of enzyme and substrate, but in the absence of any added diphosphopyridine nucleotide), which persists even after prolonged dialysis, has hitherto interfered with the use of a glycolyzing system for such determinations. We have been able to overcome this difficulty by adsorbing on charcoal the nucleotide originally present in aqueous muscle extracts. The resulting filtrate shows no glycolytic activity in the absence of added diphosphopyridine nucleotide, but contains, unimpaired, the necessary specific enzymes.

Reagents—

NaHCO$_3$ (0.154 M), saturated with 5 per cent CO$_2$:95 per cent N$_2$.
Na$_2$HAsO$_4$ (0.003 M).

Sodium hexose diphosphate (approximately 0.016 M; 1 mg. of P per cc.).

The solution is prepared from calcium hexose diphosphate by a modification of Neuberg and Sabetay's method (10), as follows: About 700 mg. of the calcium salt are added to 40 cc. of a 1 per cent solution of oxalic acid. After shaking, the mixture is neutralized with NaHCO$_3$ with chlor-phenol red as indicator. It is decolorized with charcoal and filtered. The filtrate is tested for oxalate which, if present, is precipitated with small amounts of solid calcium hexose diphosphate, the solution being refiltered through the original filter paper. The phosphorus content of the final filtrate is determined, and the solution diluted so as to contain 1 mg. of organic P per cc. Inorganic phosphorus amounting to 3 to 4 per cent may be present, but need not be eliminated. This solution, when kept in the refrigerator, is stable for several months.

Muscle Extract—The muscle acetone powder is prepared by a modification of the procedure described by Meyerhof and Kiessling (11); the aqueous extract of the powder is dialyzed and then shaken with charcoal to adsorb nucleotides.

The muscles from the hind legs and back of a cat are freed from fascia as far as possible, homogenized thoroughly in the cold with an equal weight of water and crushed ice, and centrifuged. (Although we use a Waring blendor and Sharples centrifuge for these steps, extracts of nearly the same strength can be obtained by grinding the muscle, and centrifuging or pressing off through muslin.) To the resultant solution, 4 volumes of ice-cold acetone are added in a slow stream, with stirring. After the precipitate has been allowed to stand for 30 minutes in the cold, the supernatant liquid is decanted, and the remainder centrifuged, washed twice with cold acetone in the centrifuge, transferred to a Buchner funnel, and washed with acetone and ether. After the cake is broken up into small fragments, the material is dried overnight *in vacuo* over H_2SO_4. This is the *acetone powder*.

A homogeneous paste of acetone powder is made by grinding 600 mg. with successive 2 cc. portions of water until 20 cc. have been added. After centrifugation, the clear supernatant liquid is dialyzed in the cold against running, distilled water for 24 hours and centrifuged.

To the dialyzed solution, 600 mg. of charcoal are added and the mixture is shaken mechanically in the cold for 12 to 15 hours. After centrifugation and filtration, the light brown solution is ready for use. This is the *muscle extract*.

Technique—The determinations are carried out in conventional Warburg manometers equipped with conical flasks which carry a side arm. The main vessel contains 0.4 cc. of $NaHCO_3$ (0.154 M, saturated with 5 per cent CO_2:95 per cent N_2), 0.6 cc. of Na hexose diphosphate (approximately 0.016 M), 0.3 cc. of Na_2HAsO_4 (0.003 M), and 1.0 cc. of muscle extract, equivalent to 30 mg. of acetone powder.

The diphosphopyridine nucleotide solution (0.1 to 0.5 cc.) to be tested is placed in the side arm, and water added to the vessel to make a total liquid volume of 3.0 cc. After the manometers have been filled with 5 per cent CO_2:95 per cent N_2, and the contents equilibrated for 15 minutes at 38°, the contents of the main vessel and the side arm are mixed, and shaking is continued at 100 oscillations per minute. After 5 minutes are allowed to elapse for the restoration of equilibrium, manometer readings are taken at 5 minute intervals for at least 30 minutes. The volume of CO_2

evolved, in c.mm., is plotted against time, and the slope of this curve used in the computation of the diphosphopyridine nucleotide concentration of the test solution. The rate of CO_2 evolution is constant for at least 30 to 35 minutes.

The estimation of diphosphopyridine nucleotide in biological material requires the previous preparation of blood or tissue extracts with hot water according to the method described in the accompanying paper (12).

Results and Comment

It is known that the breakdown of glycogen to lactic acid in muscle extract involves the intervention of adenylic acid and its phosphorylated derivatives, as well as phosphocreatine. In the present method, only certain intermediary steps of this conversion have been utilized; namely, the breakdown of hexose diphosphate, in the presence of arsenate, into glycerophosphoric acid and an equilibrium mixture of 3-phosphoglyceric acid, 2-phosphoglyceric acid, and phosphopyruvic acid. In the light of recent findings (13), the reactions involved in the test system are believed to be the following.

(1)   Hexose diphosphate $\xrightarrow{\text{Enzyme 1}[1]}$ 2 phosphoglyceraldehyde[2]
(2)   Phosphoglyceraldehyde + H_3AsO_4 → arsenophosphoglyceraldehyde
(3)   Arsenophosphoglyceraldehyde + DPN $\xrightarrow{\text{Enzyme 2}[1]}$
                arsenophosphoglyceric acid + DPN-H_2
(4)   Arsenophosphoglyceric acid → 3-phosphoglyceric acid[3] + H_3AsO_4
(5)   Phosphoglyceraldehyde + DPN-H_2 $\xrightarrow{\text{Enzyme 3}[1]}$
                glycerophosphoric acid + DPN
(6)   Hexose diphosphate → 3-phosphoglyceric acid[3] +
                glycerophosphoric acid

The over-all reaction, No. 6, indicates that acid is produced by the degradation of hexose diphosphate. A strictly stoichiometric relationship between the amount of substrate broken down and CO_2 evolved cannot be formulated, since the two dissociation

---

[1] Enzyme 1 represents zymohexase (14), Enzyme 2 the oxidizing enzyme of fermentation (13), and Enzyme 3 the reducing enzyme of fermentation (15)

[2] In equilibrium with dihydroxyacetone phosphate (16).

[3] In equilibrium with 2-phosphoglyceric and phosphopyruvic acids (17).

constants for phosphoric acid and the dissociation constant for the carboxyl group vary appreciably among the three acids in the equilibrium mixture (18). The amount of CO_2 liberated would depend upon the relative amounts produced of these acids. However, the constancy of the results presented below shows that the same state of equilibrium is always reached under the conditions of the test. As long as diphosphopyridine nucleotide is present in suboptimal amounts, the rate of CO_2 production depends upon the concentration of the coenzyme only.

Enzyme System—The use of charcoal to remove diphosphopyridine nucleotide from enzyme preparations has previously been reported (19). About 40 per cent of the protein in the aqueous

TABLE I

Activity of Aqueous Dialyzed Muscle Extract before and after Adsorption with Charcoal

The test system as described in the text was used   The figures indicate c.mm. of CO_2 evolved in 20 minutes.

Experi- ment No.	Diphospho- pyridine nucleotide per vessel	Muscle extract				
		Before adsorption		After adsorption		Adsorption time
		Blank	Plus nucleotide	Blank	Plus nucleotide	
	γ					*hrs.*
1	1.70	36.5	64.0	0.0	27.0	12
2	1.95	81.0	112.0	3.0	33 0	6
3	3.90	81.0	143.0	3.0	63.0	6

muscle extract (as judged by total nitrogen determinations) is lost during the treatment with charcoal. However, the enzymes active in the particular phases of carbohydrate degradation utilized in the present method are not affected by this procedure. Table I shows the effect of adding equal amounts of diphosphopyridine nucleotide to aliquots of a muscle extract, a portion of which had been adsorbed with charcoal. It is seen that the net rate, *i.e.* the rate above the blank, is approximately the same in the adsorbed and unadsorbed fractions.

If the time allowed for shaking the enzyme preparation with charcoal is 6 instead of 12 hours, a small blank amounting to 9 to 10 c.mm. of CO_2 per hour is obtained. However, when allow-

ance is made for this amount, the results obtained in assays for added nucleotide agree with those in which the blank is zero. Thus, Experiments 2 and 3, Table I, have been performed with a muscle extract which has been adsorbed on charcoal for 6 hours. For some unexplained reason, charcoal adsorption which is not preceded by dialysis frequently yields extracts which show a steady rate for only 10 to 15 minutes.

The acetone powder is very stable when kept *in vacuo* in the cold. One preparation has been kept for 4 months and showed the same activity at the end of this period as when freshly prepared. The activity of the aqueous extract remains constant for 4 to 5 days when kept in the cold. Different acetone powders

TABLE II

Constancy of Manometric Test for Diphosphopyridine Nucleotide

The test system as described in the text was used.

Experiment No.	Diphosphopyridine nucleotide per vessel	CO_2 evolution	Min.	CO_2 per γ diphosphopyridine nucleotide per hr.
	γ	*c.mm.*		*c.mm.*
1	0.87	7.0	10	48.7
2	0.47	5.7	15	48.6
3	2.36	28.7	15	48.7
4	1.69	15.0	20	48.9
5	3.80	44.2	15	46.4
6	1.09	34.5	40	47.1
7	1.05	22.5	25	51.4

Three different acetone powders were used in Experiments 1 to 4, 5 and 6, and 7, respectively.

show slight differences in activity among themselves and should be calibrated individually with solutions of known diphosphopyridine nucleotide content. Muscle extracts from the same acetone powder have equal activity. Table II shows results representative of many experiments carried out over a period of more than 6 months. Three different acetone powders were used in Experiments 1 to 4, 5 and 6, and 7, respectively. The stability of the acetone powders is demonstrated in Experiments 1 and 4, which were carried out 19 weeks apart. The experiments reported in Table II do not include those of Tables I and III which show the same constancy.

Charcoal—Different samples of charcoal were found to behave differently in their ability to yield clear muscle extracts.   In most of the experiments reported here, norit charcoal was used successfully.   Charcoal which yields dark solutions on prolonged centrifugation is to be avoided.

Arsenate—The amount of arsenate used in the test is sufficient to provide for the maximum effect (Table III).   Small variations in the concentration of As do not affect the rate of CO_2 production noticeably, while inhibition takes place to some extent only with much larger amounts (of the order of 10 times the concentration employed here).

Gas—Although 5 per cent CO_2:95 per cent N_2 with $NaHCO_3$ is used to buffer the reacting system of the present method at approximately pH 7.3, 5 per cent CO_2:95 per cent O_2 can also be

TABLE III

Effect of Varying Amounts of Arsenate on Test System for Diphosphopyridine Nucleotide

The test system as described in the text was used, except for different amounts of arsenate (Na_2HAsO_4, 3×10^{-3} M); diphosphopyridine nucleotide, approximately 1.1 γ per vessel.

Arsenate, cc	0.1	0.2	0.3	0.4
CO_2 evolved in 25 min., *c.mm.*	15.5	22.0	23.0	23.2

used.   It has been noted in the fermentation test (20) that no respiration takes place, and this is equally true for the present reaction.

Addition of Other Components of Glycolytic System—Adenosine triphosphate and inorganic phosphate, the latter in concentrations of the same order of magnitude as that of arsenate, have no effect on the rate of CO_2 production.   Higher concentrations of inorganic phosphate cause a marked inhibition.   If one accepts Warburg and Christian's (13) explanation of the mechanism of the reactions involved, it would seem that phosphate competes with arsenate in Reaction 2, resulting in arsenophosphoglyceraldehyde.

Addition of magnesium (0.2 cc. of $MgCl_2$, 0.1 M) causes the CO_2 evolution to diminish after 20 minutes, and to cease after 35 to 45 minutes; while in its absence the reaction continues, though at a lower rate, even after 3 hours.   Increasing the arsenate con-

centration overcomes this inhibition. It is probable that magnesium arsenate is precipitated in this mixture.

Fig. 1 shows a typical calibration curve, which was obtained with Preparation B, the standard preparation of the preceding paper, as the source of diphosphopyridine nucleotide. The rate of CO_2 evolution increases linearly with the amount of nucleotide present up to 5 γ of diphosphopyridine nucleotide. With higher amounts, a linear relation is no longer obtained.
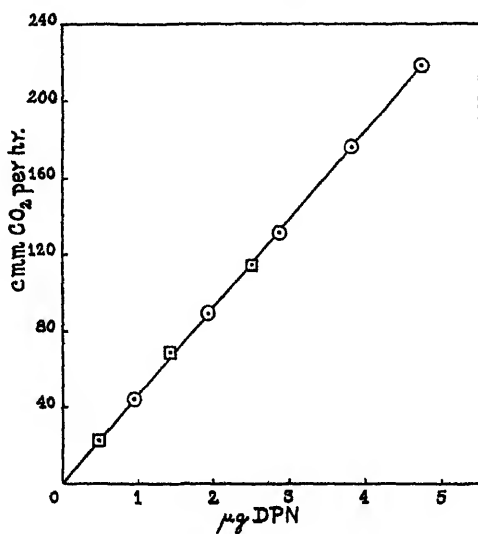
FIG. 1. The amount of diphosphopyridine nucleotide in micrograms added to the test system is plotted against the rate of CO_2 evolution in c.mm. per hour. The source of nucleotide was an aqueous solution of a preparation of known purity. The results were obtained in two different experiments as indicated by the squares and circles.

SUMMARY

A method is described for the manometric estimation of diphosphopyridine nucleotide (cozymase) in aqueous solutions containing 1 to 10 γ of nucleotide per cc. The method is based on the catalysis by diphosphopyridine nucleotide of the breakdown of hexose diphosphate in the presence of arsenate and an aqueous muscle extract freed from nucleotide by charcoal adsorption as the source of required enzymes.

BIBLIOGRAPHY

1. von Euler, H., Adler, E., and Hellstrom, H., *Z. physiol. Chem.*, **241**, 239 (1936).
2. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
3. Green, D. E., and Dewan, J. G., *Biochem. J.*, **31**, 1069 (1937).
4. Rivers, T. M., *Bull. Johns Hopkins Hosp.*, **33**, 149, 429 (1922).
5. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 352, 360 (1937).
6. Kohn, H. I., *Biochem J.*, **32**, 2075 (1938). Vilter, R. W., Vilter, S. P., and Spies, T. D., *J. Am. Med. Assn.*, **112**, 420 (1939).
7. Myrbäck, K., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **2**, 139 (1933). von Euler, H., *Ergebn. Physiol.*, **38**, 1 (1936).
8. Schlenk, F., and Vowles, R. B., *Ark. Kemi, Mineral. o. Geol.*, **13 B**, No. 19 (1940).
9. von Euler, H., Albers, H., and Schlenk, F., *Z. physiol. Chem.*, **240**, 113 (1936).
10. Neuberg, C., and Sabetay, S., *Biochem. Z.*, **161**, 240 (1925).
11. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **283**, 83 (1935).
12. Hutchens, J. O., Jandorf, B. J., and Hastings, A. B , *J. Biol. Chem.*, **138**, 321 (1941).
13. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
14. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934); **273**, 413 (1934). Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108 (1940).
15. Negelein, E., and Bromel, H., *Biochem. Z.*, **303**, 231 (1939).
16. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **279**, 40 (1935).
17. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **280**, 99 (1935).
18. Kiessling, W., *Biochem. Z.*, **273**, 103 (1934).
19. Green, D. E., Needham, D. M., and Dewan, J. G., *Biochem. J.*, **31**, 2327 (1937).
20. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 77 (1939).

SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE BY CHILOMONAS PARAMECIUM

By JOHN O. HUTCHENS,* BERNHARD J. JANDORF, and A. BAIRD HASTINGS

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

Chilomonas paramecium is a cryptomonad flagellate capable of growing in the dark, utilizing ammonia as a source of nitrogen and a variety of compounds including acetate, formate, or CO_2 as a source of carbon (1). Other than the general fact that it synthesizes starch, fat, and protein (1), little is known of the actual compounds synthesized by the organism, or of the metabolic processes by which it obtains the energy necessary for the requisite syntheses. It was felt that identifying and measuring the amount of a respiratory catalyst of known structure and function in chilomonads would serve the twofold purpose of contributing knowledge concerning both of the above questions.

Of the cellular oxidation systems, those involved in carbohydrate metabolism are perhaps best known, and the important rôle of the pyridine nucleotides has been repeatedly demonstrated. Diphosphopyridine nucleotide (cozymase) is known to be widely distributed in animal and vegetable cells, and adequate methods for its determination are available. Experiments were, therefore, conducted with the purpose of measuring the diphosphopyridine nucleotide content of chilomonads taken from cultures in which carbon was supplied as acetate and nitrogen was furnished in the form of ammonia.

Materials and Methods

The organisms were grown in sterile, pure, mass cultures in 125 cc. Erlenmeyer flasks containing 50 cc. of the culture solution described in Table I.

---

* Fellow of the National Research Council, Division of Biological Sciences.

The cultures were inoculated with about 1000 organisms per cc. and allowed to develop for the desired periods at 26° ± 0.2°. Under these conditions, the organisms multiplied at the rate of about 3.3 divisions per day until a population of about 200,000 organisms per cc. was reached after about 65 hours. Growth stopped at this time because of increasing alkalinity of the culture.

The organisms chosen for an experiment were concentrated by gentle centrifugation (200 to 300 g) and aliquots were withdrawn for ascertaining the number of organisms per cc. (by hemocytometer count) and the wet and dry weights of the cells.

The organisms used for the determination of diphosphopyridine nucleotide content were again concentrated by centrifugation, the supernatant fluid withdrawn, and the material was then evapo-

TABLE I

Composition of Solution Used for Culturing Chilomonads

Original pH 6.5.

	mg. per cent	mM per l.
$NaC_2H_3O_2 \cdot 3H_2O$.	244	18 3
NH_4Cl..	46	8.60
$(NH_4)_2SO_4$	10	0.757
K_2HPO_4	150	8.62
$MgCl_2$..	1.0	0.105
$CaCl_2$...	1.0	0.090
$FeCl_3 \cdot 6H_2O$	0.17	0.00625
Thiamine hydrochloride	0 01	

rated to dryness in a boiling water bath for 12 minutes. The dry material was resuspended by grinding with a small amount of sand in a known volume of $NaHCO_3$ (11.5 mM per liter) equilibrated with 5 per cent CO_2 in N_2. The suspension was centrifuged and the clear supernatant solution used for measuring the nucleotide content. This was carried out by comparing the rate of CO_2 evolution catalyzed by aliquots of the extracts with that catalyzed by known amounts of diphosphopyridine nucleotide, according to the procedure described in an accompanying paper (2).

In preliminary experiments, the pH of the culture solution varied, being 6.8 for cultures 48 hours old, and 7.2 to 7.5 for those 72 hours old. It was found that during heat treatment losses of

nucleotide occurred which varied with the pH of the material. In subsequent experiments, sufficient HCl was added to bring the solution to pH 6.0 before evaporation. The method of correcting for this effect is discussed below.

Results

The results of several experiments are presented in Table II. Experiments 3a and 3b demonstrate the variation in amount of nucleotide found in two samples of the same suspension of organisms when one sample was evaporated at pH 6.0 and the other at pH 7.5. The average of the values obtained (Column 8), exclu-

TABLE II

Diphosphopyridine Nucleotide Content of Chilomonas

						Diphosphopyridine nucleotide		
Experiment No.	Age of culture	pH of culture	No. of cells per cc.	Wet weight of cells	Dry weight of cells	Per mg wet weight	Per mg. dry weight	Corrected,* per mg. dry weight
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	hrs.		$\times 10^8$	*mg. per cc.*	*mg. per cc.*	γ	γ	γ
1	48	6.8	1.08	2.68	0 62	0.157	0.68	0.91
2	72	6 8	1.20	2.99	0.66	0 141	0.63	0.84
3a	72	7.5	1.01	2.89	0.77	0.138	0.52	0.84
3b	72	6 0	1.01	2.89	0.77	0.185	0.69	0.87
4	48	6 0	1.15	2.43	0.63	0.165	0.64	0.81
5	48	6.0	1.19	3.00	0.75	0.167	0.67	0.85
6	72	6.0	2.70	6.71	1.53	0.144	0.63	0.80

* Corrected for inactivation at the stated pH from the data in the text.

sive of Experiment 3a, is 0.66 γ of diphosphopyridine nucleotide per mg. of dry cells. The rather wide variation in values per mg. of wet cells (Column 7) is probably the result of the difficulty experienced in removing all of the supernatant solution from the capillary into which the cells were packed by centrifugation.

In order to correct the results in Column 8 for the amount of nucleotide destroyed during heat treatment, aliquots of a solution of diphosphopyridine nucleotide dissolved in the culture medium, adjusted to the desired pH, were subjected to the same conditions as those employed in the preparation of *Chilomonas* extracts (12 minutes in a boiling water bath). The activity of these samples

was then compared with that of other unheated aliquots. It was found that the per cent inactivation was 21 at pH 6.0, 25 at pH 6.8, and 38 at pH 7.5. By applying the correction for inactivation at the appropriate pH to the data of Column 8, Table II, we obtained the values of Column 9. This column gives the values of diphosphopyridine nucleotide presumably present originally in the organisms, expressed as micrograms per mg. of dry weight.

The average value obtained for all the experiments presented is 0.85 γ of diphosphopyridine nucleotide per mg. of dry cells. No significant differences were found in the nucleotide content of the organisms from cultures of different ages, the average value for cells from 48 hour and 72 hour cultures being 0.86 and 0.84 γ of nucleotide per mg. of dry cells, respectively.

DISCUSSION

It should be noted that in the procedure as described only the oxidized coenzyme is estimated, the dihydro form being destroyed by heating to 100° in acid solution (3). That the amount of the latter is by no means negligible has been shown for yeast (4), in which the ratio oxidized to reduced diphosphopyridine nucleotide is approximately 1 in either the actively respiring or dormant cell. It may well be that similar conditions obtain in *Chilomonas*. However, since most of the values reported in the literature are in terms of oxidized rather than total nucleotide (5), our values may be compared with most other published results.

From Myrbäck's compilation of data (5), it may be seen that the nucleotide content of many fresh yeasts is of the same order of magnitude (0.9 to 1.0 γ per mg. of dry weight) as that reported here for *Chilomonas*, though much higher values (up to 3.5 γ) have been found in some yeast strains.

It is interesting to note that the nicotinic acid content of *Chilomonas* has been found in one instance (Experiment 6, Table II) to be 0.239 γ per mg. of dry cells. (This determination was kindly carried out by Dr. E. H. Stotz, according to the CNBr method, as modified by Melnick and Field (6).) The nicotinic acid content of the determined nucleotide accounts for approximately 65 per cent of this value. It is possible that a portion or all of the remaining nicotinic acid is present in the form of triphosphopyridine nucleotide or dihydrodiphosphopyridine nucleotide or both. It

would seem, therefore, that *Chilomonas* has the ability to synthesize nicotinic acid and diphosphopyridine nucleotide even when grown in a medium devoid of pyridine compounds.

SUMMARY

The diphosphopyridine nucleotide content of *Chilomonas paramecium* has been measured manometrically and found to be $0.85 \pm 0.06 \gamma$ per mg. of dry cells. The synthesis of nicotinic acid and diphosphopyridine nucleotide is accomplished by the organisms in a solution containing ammonia as the only nitrogen source and acetate as the sole source of carbon.

BIBLIOGRAPHY

1. Mast, S. O , and Pace, D. M., *Protoplasma*, **20**, 326 (1933).
2. Jandorf, B. J., Klemperer, F. W., and Hastings, A. B., *J. Biol. Chem.*, **138**, 311 (1941).
3. Sula, J., *Ark. Kemi, Mineral. o. Geol.*, **12 B**, No. 28 (1936).
4. Adler, E., and Calvet, F., *Ark. Kemi, Mineral. o. Geol* , **12 B**, No. 32 (1936)
5. Myrback, K., in Junk, W , Oppenheimer, C., and Weisbach, W., Tabulae biologicae, The Hague, **14**, 110 (1937).
6. Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, **134**, 1 (1940).

CARBOXYLASE

By D. E. GREEN,* D. HERBERT, and V. SUBRAHMANYAN

(*From the Biochemistry Department, University of Cambridge, Cambridge, England*)

Neuberg and Rosenthal (1) in 1913 discovered in yeast an enzyme which catalyzes the decarboxylation of pyruvic acid with formation of acetaldehyde. Lohmann and Schuster (2) some 24 years later showed that diphosphothiamine is an essential component of the enzyme system. Some attempts (3–8) have been made to purify the enzyme.

The present communication deals with the purification and properties of carboxylase. The enzyme was isolated from top brewers' yeast as a diphosphothiamine-magnesium-protein.

Method of Testing and Preparation

We have defined as the unit of carboxylase activity an amount of enzyme which catalyzes the production of 100 c.mm. of CO_2 in 3 minutes at 30°. The test mixture (volume 3.3 cc.) contained 0.5 cc. of m pyruvate and 0.3 cc. of 0.5 m citrate buffer, pH 6.0.

The light absorption ($\log I_0/I$) at 280 mμ in a 2 cm. cell was used as a measure of the concentration of protein, and the activity ratio, $\log I_0/I$ to units per cc., is used throughout this paper as an index of the purity level of the enzyme.

1. Fresh brewers' ale yeast is mixed with 5 volumes of tap water and filtered with suction. The cake is broken up finely and dried at room temperature in a brisk current of air.

100 gm. of dried yeast are slowly added with stirring to 300 cc. of m/15 phosphate buffer, pH 7.2, and incubated 1 hour at 37°. The mixture is diluted with 400 cc. of water and centrifuged.

---

* Beit Memorial Research Fellow.

Present address of D. E. Green, Department of Biological Chemistry, Harvard Medical School, Boston.

The supernatant fluid (530 cc.) contains 9650 units at the activity ratio 21.8.

2. The solution is mixed in order with 80 cc. of 0.5 M phosphate buffer, pH 7.2, and 40 cc. of M calcium acetate. The precipitate is centrifuged off and washed with $1\frac{1}{2}$ volumes of water. The supernatant fluid and wash fluid combined (700 cc.) contain 8170 units at the activity ratio 17.6.

3. 38 gm. of ammonium sulfate (analytical reagent grade only) are added to each 100 cc. of solution. The precipitate can be packed tightly only by prolonged centrifugation (at least 1 hour).

TABLE I

Results of Fractionations with Ammonium Sulfate

Fraction No.	Saturated ammonium sulfate added	Degree of saturation	Total units	Activity ratio
	volume			
I	0.35	0.35	168	13.0
II	0.52	0.42	960	1.79
III	0.88	0.52	4450	1.22
IIIa	0.44	0.47	940	1.15
IIIb	0.55	0.52	2060	0.90
IIIc	0.77	0 58	1270	1.34
IIIb$_1$	0.41	0.47	440	0.89
IIIb$_2$	0.58	0.53	587	0.78
IIIb$_3$	1.0	0.63	116	1.25

The precipitate is dissolved by addition of 300 cc. of 0.04 M citrate buffer, pH 6. The solution (344 cc.) contains 7250 units at the activity ratio 7.1.

4. 38 gm. of ammonium sulfate are added for each 100 cc. of buffer solution. The precipitate after hard packing is dissolved by addition of 126 cc. of 0.04 M citrate buffer, pH 6. The solution (162 cc.) contains 6300 units at the activity ratio 3.0 and is 0.11 saturated with respect to ammonium sulfate.

5. Fractionation with ammonium sulfate leads to the distribution of the enzyme shown in Table I, Fractions I, II, and III. Fractions I and II are discarded. Fraction III is suspended in 100 cc. of a solution made up of equal parts of saturated ammonium sulfate and 0.1 M citrate buffer, pH 6.0. On addition of 100 cc. of water the protein precipitate is dissolved. The solution

now 0.25 saturated with respect to ammonium sulfate is submitted to the second fractionation (Table I, Fractions IIIa, b, and c). Fraction IIIb is treated as described above for Fraction III and submitted to the third fractionation (Fractions IIIb$_1$, b$_2$, and b$_3$, Table I). We have been unable to advance the purity of the enzyme beyond the activity ratio 0.78, although the preparation at this stage is not homogeneous.

After step (3) of the purification procedure the enzyme is stable. But it is essential to reach that stage with the minimum of delay. Ammonium sulfate solutions of the enzyme left at 0° or at room temperature retain their activity unimpaired for weeks. We have adopted the practice of keeping the enzyme suspended in half saturated ammonium sulfate solution. The enzyme is added to the reaction mixture in the form of a suspension and is in solution only during the manometric run.

Properties of Purified Enzymes

At the activity ratio 0.8, 1 mg. of the protein is equivalent to 6.07 enzyme units. The Q_{CO_2} (c.mm. of CO_2 per hour per mg. of dry weight) is therefore $6.07 \times 100 \times 60/3$ or 12,140 at 30°. A solution containing 1 mg. per cc. has a log I_0/I of 4.87 at 280 mμ in a 2 cm. cell.

The presence of diphosphothiamine in the enzyme can be shown as follows: The protein-free extract of the boiled or acidified enzyme is treated with yeast phosphatase and the thiamine thereby formed is oxidized to thiochrome with ferricyanide. Dr. Y. L. Wang of the Dunn Nutritional Laboratory kindly carried out for us the estimations of diphosphothiamine by the thiochrome fluorescence method (9); e.g., in a solution containing 15.9 units per cc. 12 γ of diphosphothiamine were found. When the phosphatase treatment was omitted, the thiochrome test was practically negative, indicating that the thiamine present in the enzyme was exclusively in the form of diphosphothiamine. Since 15.9 enzyme units are equivalent to 2.62 mg. of protein, the percentage of diphosphothiamine at the activity ratio 0.8 is $12/2620 \times 100$ or 0.46.

The presence of magnesium in carboxylase at the activity ratio 0.8 was demonstrated as follows: An ammonium sulfate-citrate solution of the enzyme containing 33 mg. of dry weight of protein

was ashed.   The ash was dissolved in dilute HCl, and oxalic acid added to remove calcium.   No calcium was found.   Magnesium was precipitated as $MgNH_4PO_4$ and estimated as inorganic phosphate.   Analyses showed that the magnesium content of the solution was 42.8 γ.   The percentage of magnesium in carboxylase at the activity ratio 0.8 is therefore 42.8/33,000 or 0.13. A control was carried out on the ammonium sulfate-citrate solution in which the enzyme was dissolved.   No trace of magnesium was found in the same volume of solution as that in which the enzyme was suspended.   Magnesium estimations were carried out on salt solutions of the enzyme, since the enzyme dissociates into its component parts on prolonged dialysis, and both diphosphothiamine and the metal diffuse out of the dialysis sac.   Negative tests were given by the purified enzyme solution for the following metals: Ca, Mn, Ni, Cr, Cu, and Fe.

Diphosphothiamine shows an absorption band at 266 mμ. No band at this wave-length can be detected in the spectrum of carboxylase.   It can be calculated, however, that the absorption at 266 mμ due to diphosphothiamine is inadequate to influence the shape of the absorption curve.   A solution containing 1 mg. of purified enzyme per cc. has an extinction of 2.15 at 266 mμ.   This amount of enzyme contains 4.6 γ of diphosphothiamine which has an extinction of 0.09 (10).

Kinetics—The maximum velocity of carboxylase is attained at about M/6 concentration of pyruvate (*cf.* Fig. 1).   The half speed concentration is about M/33.   It will be observed that in the conditions for the standard activity test the concentration of pyruvate was just sufficient to saturate the enzyme.

The proportionality between the reaction velocity and the amount of enzyme holds over the limited range of enzyme concentration employed in the manometric test (*cf.* Fig. 2).

Table II shows the dependence of carboxylase activity on the pH of the medium.   The nature and concentration of the buffer are as important as the hydrogen ion concentration (*cf.* Table III). The conditions of pH and concentration of citrate buffer used in the standard test yield higher carboxylase activity than has been obtained with any other set of conditions.

Substrate Specificity—Carboxylase decarboxylates other α-ketonic acids besides pyruvic (*cf.* Table IV).   α-Ketoisovaleric
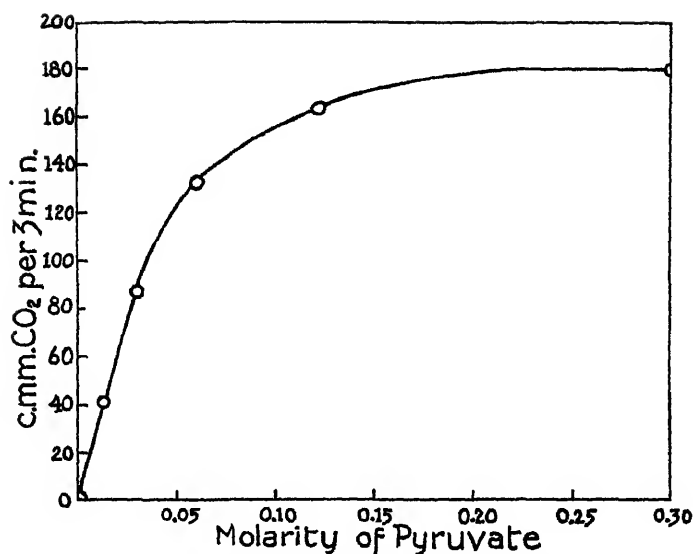
Fig. 1. Effect of pyruvic acid concentration on carboxylase activity. Conditions, 0.3 cc. of 0.5 M citrate buffer, pH 6.0, final volume 3.3 cc., and temperature = 30°.
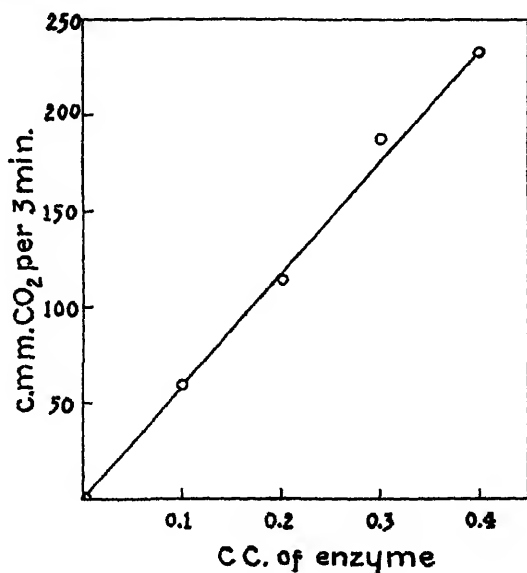
Fig. 2. Proportionality between CO_2 evolution and enzyme concentration. Conditions, 0.3 cc. of 0.5 M citrate buffer, pH 6.0, 0.5 cc. of M pyruvate, final volume 3.3 cc., and temperature = 30°.

acid is decarboxylated at about the same velocity as pyruvic acid, whereas the next higher homologue, α-ketoisocaproic acid, is attacked at one-twenty-fifth the rate. Oxaloacetic acid shows considerable activity, whereas the next higher homologue, α-ketoglutaric acid, is practically inactive as a substrate. The introduction of a phenyl group as in phenylpyruvic acid and p-hydroxyphenylpyruvic acid leads to inactivity. Acetoacetic acid (β-ketonic acid) shows no activity with the enzyme. The split

TABLE II

Variation of Carboxylase Activity with pH of Medium

Each manometer vessel contained 0.3 cc. of enzyme solution, 0.3 cc. of M pyruvate, and 0.3 cc. of 0.5 M buffer. The total volume in each case was 3.3 cc. Temperature 30°. The values are expressed in c.mm. of CO_2 per 3 minutes.

Acetate buffer		Citrate buffer, pH 6.0	Phosphate buffer	
pH 4.7	pH 5.3		pH 6 5	pH 7.2
49	125	161	72	14

TABLE III

Effect of Chemical Nature and Concentration of Buffer on Carboxylase Activity

Each manometer vessel contained 0.3 cc. of enzyme and 0.5 cc. of M pyruvate in a total volume of 3.3 cc. 0.5 M citrate and phosphate buffers, pH 6.0, were used.

	Citrate buffer			Phosphate buffer		
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
Buffer added, cc........ ...	0.3	1.0	2.0	0.3	1.0	2.0
CO_2 per 3 min., c.mm.... .	183	174	119	127	100	56

enzyme does not decarboxylate those substrates which are active with the intact enzyme except in presence of diphosphothiamine and magnesium.

Inhibitors—The effects of various inhibitors have been tested on the purified enzyme (*cf.* Table V). 0.0001 M concentrations of silver, copper, and mercury completely inhibit activity. Acetaldehyde has only a slight depressing effect, whereas iodoacetic acid, lactic acid, and arsenious acid have no effect.

Reversible Resolution—Carboxylase occurs in yeast as a conjugated protein and it has been isolated as such. At no stage in the purification process was it possible to increase the activity of the preparation by addition of diphosphothiamine and magnesium. Furthermore the fact that all the units could be accounted for from the initial crude extract to the final stage of

TABLE IV

Decarboxylation of α-Ketonic Acids by Yeast Carboxylase

Substrate, 0.04 M	CO$_2$
	c.mm.
Pyruvic acid...	596
α-Ketoisovaleric acid...	525
Oxaloacetic acid..	191
α-Ketoisocaproic acid....	24
α-Ketoglutaric acid	8
Phenylpyruvic "	0
p-Hydroxyphenylpyruvic acid.	0
Acetoacetic acid........	0
No addition.....	0

TABLE V

Inhibitors of Carboxylase Activity

Reagent	Final concentration	Per cent inhibition
AgNO$_3$	M/10,000	100
CuSO$_4$.....	M/10,000	100
Hg(NO$_3$)$_2$...	M/10,000	100
ZnSO$_4$.....	M/10,000	0
Acetaldehyde	M/65	21
"	M/160	11
Iodoacetic acid	M/65	0
Caprylic alcohol. .	Saturated solution	31

purity rules out any significant dissociation of carboxylase. Our experiments suggest that in slightly acid condition (pH about 6) carboxylase does not dissociate appreciably, whereas on the alkaline side (pH 8) dissociation is considerable. There is one apparent exception. A solution of carboxylase which has been exhaustively dialyzed against distilled water (final pH about 5.5)

loses both diphosphothiamine and magnesium, indicating that carboxylase in salt-free solution at slightly acid pH must dissociate to some degree. This conclusion, however, cannot be drawn on the basis of the dialysis experiment alone, since coincident with the dialysis the residual protein becomes inactive. The fact that at no stage in the dialysis procedure is it possible to increase activity by addition of the two missing components would suggest that denaturation precedes dissociation.

In ammoniacal ammonium sulfate solution carboxylase is resolved into its component parts. The protein moiety can be precipitated out by raising the salt concentration, whereas diphosphothiamine and magnesium remain in the solution. This resolution involves no loss in the activity of the protein. The recombination of protein, diphosphothiamine, and magnesium is not instantaneous, and the velocity depends upon factors such as concentration of reactants, order of mixing, etc.

Preparation of Split Enzyme—To 1 volume of the enzyme solution are added 2 volumes of water and 4 volumes of ammoniacal ammonium sulfate solution (9 parts of saturated ammonium sulfate solution and 1 part of concentrated NH_3). The precipitate is centrifuged sharply and dissolved in water. The process is repeated three times in all. The precipitate is finally suspended in a solution consisting of equal parts of saturated ammonium sulfate solution and 0.5 M citrate buffer, pH 6.0. The suspension whether concentrated or dilute is stable at room temperature over a period of weeks.

Kinetics of Recombination—With a given amount of enzyme and metal the decarboxylation reaction is proportional to the concentration of diphosphothiamine within certain limits (*cf.* Fig. 3). The form of the curve would suggest that carboxylase exists largely in the dissociated form under the conditions of the experiment. However, this simple interpretation can be shown to be untenable. In the above experiment 1 γ of diphosphothiamine produced 90 c.mm. of CO_2 per 3 minutes which was roughly one-fifth the maximum CO_2 production which the specific protein could catalyze in the presence of excess diphosphothiamine. Thus clearly the specific protein was not saturated by 1 γ of diphosphothiamine. We should expect therefore that in consequence either of halving or doubling the concentration of protein the

CO_2 production induced by 1 γ of diphosphothiamine would remain unchanged.   That such is not the case is shown in Table VI. The maximum effect with 1 γ of diphosphothiamine is attained with an amount of specific protein which is 3.3 as much as was used in the experiment with varying amounts of diphosphothiamine.   In other words, to obtain in reconstruction experiments the same catalytic efficiency for 1 γ of diphosphothiamine
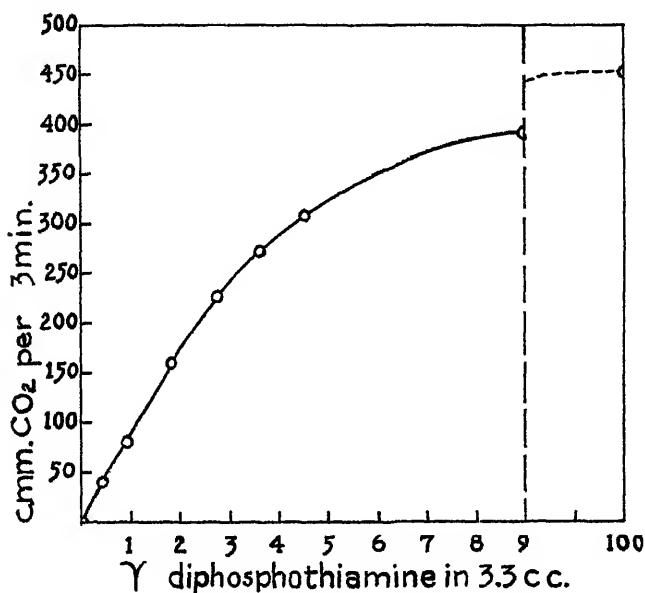
FIG. 3. Dependence of CO_2 evolution on concentration of diphosphothiamine in reconstructed system.   Conditions, 0.15 cc. of specific protein suspension, 0.3 cc. of 0 1 per cent Mn, 0 3 cc. of 0.5 M citrate buffer, pH 6.0, 0.5 cc. of M pyruvate, final volume 3.3 cc., and temperature = 30°.

as is shown in the intact enzyme 3.3 $\times$ 453/126 or 11.9 times the stoichiometric amount of specific protein must be employed. As the ratio of protein to diphosphothiamine approaches unity the catalytic efficiency of diphosphothiamine per mg. of specific protein increases.   For example with 0.5 cc. of enzyme which is 11.9 times the protein equivalent of 1 γ of diphosphothiamine the CO_2 evolution per microgram of diphosphothiamine is 126 c.mm. per 3 minutes, whereas with 0.05 cc. of enzyme which is 1.19 times

the protein equivalent of 1 γ of diphosphothiamine the CO_2 evolution per microgram of diphosphothiamine is 51 c.mm. per 3 minutes. The efficiency of diphosphothiamine per mg. of protein is therefore 10/2.47 or approximately 4 times as high in the 10-fold diluted enzyme. At the lowest dilution of enzyme tested the activity of diphosphothiamine per mg. of specific protein is about 1/3.1 the value observed in the intact enzyme.

The presence of some divalent metal such as manganese is necessary in the reconstructed enzyme system. The relation between the rate of CO_2 evolution and the concentration of manganese is shown in Fig. 4. The quantity of specific protein employed in these experiments (1.7 units) is combined in the

TABLE VI

Activity of 1 γ of Diphosphothiamine in Presence of Varying Excess of Specific Protein and Metal

The specific protein was added in the form of a suspension in 0.5 saturated ammonium sulfate solution. Additions of less than 1 cc. of specific protein were supplemented by 0.5 saturated ammonium sulfate solution to make the salt concentration identical in all manometer vessels. The additions were as follows: 0.3 cc. of 0.1 per cent Mn, 1 γ of diphosphothiamine, 0.3 cc. of 0.5 M citrate buffer, pH 6.0, 0.5 cc. of M pyruvate; total volume 3.3 cc.

Specific protein, cc.	0.05	0.15	0.25	0.50	1.0
CO_2 per 3 min. per microgram diphosphothiamine, c.mm.	51	86	106	126	129

integral enzyme with 0.36 γ of magnesium which is the equivalent of 0.82 γ of manganese. At the lower dilutions of manganese tested 10 γ are equivalent to 1.13 enzyme units. The ratio, activity in intact enzyme to activity in reconstructed enzyme, for manganese is therefore 10 $\times$ 1.7/1.13/0.82 or 18.3. Manganese is not a normal constituent of carboxylase. We have found, however, that magnesium can be qualitatively replaced by all divalent metals and quantitatively replaced by manganese.

Order of Additions—In the initial reconstruction experiments it was found that the same mixture of the three components yielded divergent results from one manometric run to another. This discrepancy was ultimately traced to variations in the order of addition of the three components. For example when the three

components were added in the order, enzyme, manganese, diphosphothiamine, the CO_2 evolution was 89 c.mm. per 3 minutes
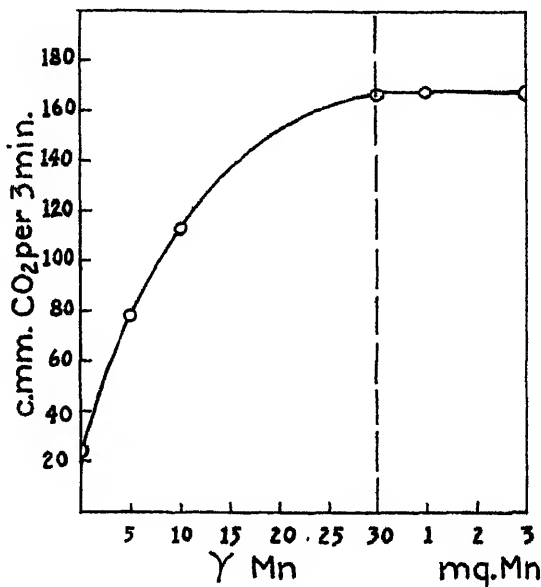
FIG. 4. Dependence of CO_2 evolution on concentration of manganese in reconstructed system. Conditions, 100 γ of diphosphothiamine, 0.5 cc. of M pyruvate, 0.3 cc. of citrate buffer, pH 6.0, final volume 3.3 cc., and temperature = 30°.

TABLE VII

Influence of Order of Additions on Reconstruction of Carboxylase Activity

Each manometer vessel contained 1 cc. of enzyme, 3 γ of diphosphothiamine, 0.3 cc. of 0.1 per cent Mn, 0.5 cc. of M pyruvate, and 0.3 cc. of 0.5 M citrate buffer, pH 6.0; total volume 3.3 cc.

Citrate and water added	CO_2 per 3 min.
	c.mm.
At once without incubation...	76
" " followed by 10 min. incubation..... ..	129
After 10 min. incubation..	184

but when diphosphothiamine was added before manganese the value was 60 c.mm. per 3 minutes. Similar effects are summarized in Table VII. The following are the conditions for

obtaining maximum activity. Enzyme, manganese, and diphosphothiamine are added in the order named. The mixture is incubated in the manometer cup at room temperature for 10 minutes. Citrate buffer and water are added and the manometric run at 30° is begun. After temperature equilibration, pyruvate is tipped in from a Keilin cup and the initial 3 minute velocity of CO_2 production is measured.

Specificity of Components—Diphosphothiamine cannot be replaced by monophosphothiamine, thiamine, flavin-adenine dinucleotide, muscle adenylic acid, or adenosine triphosphate. All the divalent metals yet tested can replace magnesium in the reconstructed system qualitatively if not quantitatively. It is of interest that iron is active in the ferrous form but practically inactive in the ferric form. Another trivalent metal tested, *viz.* aluminum, also showed practically no activity. There are some grounds for the view that the metal acts as a chemical bridge between the specific protein and diphosphothiamine.

Metal, 1 mg. per 3.3 co	No addition	Mg^{++}	Mn^{++}	Co^{++}	Cd^{++}	Zn^{++}	Ca^{++}	Fe^{++}	Al^{+++}	Fe^{+++}
CO_2, *c.mm.* ..	23	875	850	694	422	282	558	550	50	39

Note on Preparations—The sample of diphosphothiamine used in these experiments was prepared by the method of Weil-Malherbe (11). Phenylpyruvic acid, α-ketoisovaleric acid, and α-ketoisocaproic acid were prepared from their respective amino acids with the *d*-amino acid oxidase and oxygen as oxidizing agents. Oxaloacetic acid was prepared by the method of Wohl and Oesterlin (12). *p*-Hydroxyphenylpyruvic acid was kindly supplied by Dr. Hartree of the Molteno Institute.

SUMMARY

The purification and properties of carboxylase are described. At the highest purity level reached the enzyme contains 0.46 per cent diphosphothiamine and 0.13 per cent magnesium. The enzyme does not dissociate at pH 6 but dissociates above pH 8. Details are given of a method for the reversible resolution of carboxylase. The recombination of the three components to

form the original catalytically active enzyme is complex, and the factors which affect the combination have been studied in some detail.

We are indebted to Dr. E. Watchorn for her help in the estimation of magnesium, to Dr. H. Weil-Malherbe for his gift of diphosphothiamine, and to the Green King Brewery of Cambridge for a generous supply of yeast.

BIBLIOGRAPHY

1. Neuberg, C., and Rosenthal, P., *Biochem. Z.*, **51**, 142 (1913).
2. Lohmann, K., and Schuster, P., *Biochem. Z.*, **294**, 188 (1937).
3. Langenbeck, W., Juttermann, R., Schafer, O., and Wrede, H., *Z. physiol. Chem.*, **221**, 1 (1933).
4. Langenbeck, W., Wrede, H., and Schlockermann, W., *Z. physiol. Chem.*, **227**, 263 (1934).
5. Schonebeck, O., *Biochem Z.*, **272**, 42 (1934); **275**, 330 (1934–35); **277**, 451 (1935).
6. Axmacher, F., and Bergstermann, H., *Biochem. Z.*, **272**, 259 (1934).
7. Westerkamp, H., *Biochem. Z.*, **263**, 239 (1933).
8. Melnick, J. L., and Stern, K. G., *Enzymologia*, **8**, 129 (1940).
9. Wang, Y. L., and Harris, L. J., *Biochem. J.*, **33**, 1356 (1939).
10. Melnick, J. L., *J. Biol. Chem.*, **131**, 615 (1939).
11. Weil-Malherbe, H., *Biochem. J.*, **34**, 980 (1940).
12. Wohl, A., and Oesterlin, C., *Ber. chem. Ges.*, **34**, 1144 (1901)

A QUANTITATIVE METHOD FOR THE DETERMINATION OF BLOOD IN THE FECES OF SHEEP BY MEANS OF THE EVELYN PHOTOELECTRIC COLORIMETER*

By JOHN S. ANDREWS and H. J. BROOKS

(From the Department of Parasitology, Puerto Rico Agricultural Experiment Station, Río Piedras, and the Department of Chemistry, School of Tropical Medicine, San Juan, Puerto Rico)

During the summer of 1938 one of us (J. S. A.), while engaged in experimental work with sheep infected with the stomach-worm, *Haemonchus contortus*, became interested in making a study of the nature of the anemia produced by these nematodes. The examination of the feces of sheep a few days after artificial infection with stomach-worm larvae revealed the presence of blood. Quantities of blood were also found in the contents of the fourth stomachs of severely infected animals at autopsy. The absence of other lesions in the digestive tract which could have been responsible for the hemorrhage observed showed that the blood came originally from lesions produced by the nematodes in the wall of the fourth stomach. Since these observations indicated that a considerable loss of blood occurred in sheep harboring heavy stomach-worm infections, it appeared to be of interest to make a quantitative study of this loss in an attempt to ascertain its relationship to the anemia observed. To do this it was first necessary to find a method for the quantitative determination of blood in the feces of sheep.

A search of the literature revealed that van Eck (1) had published a method for the quantitative determination of blood in human feces. This method, as originally described, was found to be unsatisfactory because of the large quantities of coloring matter and plant fiber present in the feces of sheep. A crude procedure was finally developed which involved the boiling of

---

* Published with the permission of the Director of the Puerto Rico Agricultural Experiment Station of the University of Puerto Rico

sheep feces containing blood with glacial acetic acid, the treatment of the diluted extract with commercial benzidine and hydrogen peroxide, and the comparison of the intensities of the properly diluted colored solutions with appropriate standards. This method made possible a rough estimate of the quantity of blood present, but it could not be relied upon to produce consistent results. Accordingly, at the first opportunity for continuing the investigation, the problem was again attacked in an attempt to find a more satisfactory technique.

Before the present work was begun, the attention of the authors was called to two important papers. The first, by Bing and Baker (2), described a technique for the use of a purified benzidine reagent, together with a 0.6 per cent solution of hydrogen peroxide, for the quantitative determination of hemoglobin in minute quantities of blood. The second, by Bing (3), gave an account of a method for the purification of commercial benzidine and stressed the importance of the purity of that compound in quantitative hemoglobin determinations.

Although the extraction of the hemoglobin or its derivatives from the feces and the dilution of the acetic acid extract as described in the present paper were carried out very much as in 1938, the benzidine reagent used was purified according to the technique of Bing, and the feces extract containing the acid hematin was treated with benzidine and hydrogen peroxide according to the technique of Bing and Baker. The relative color intensities of the resulting solutions were then measured by means of the Evelyn photoelectric colorimeter (4).

The method for the quantitative determination of blood in the feces of sheep as it is described in the following pages is presented simply as the result of an attempt on the part of the authors to solve a definite problem in connection with the study of the hemorrhage associated with stomach-worm infection in these animals. It is hoped that the information thus made available in the literature will be of some value to other workers interested in the quantitative study of gastrointestinal hemorrhage.

Materials and Reagents—The materials and reagents necessary for carrying out the procedure described are as follows:

1. Blood-free sheep feces.
2. 50 cc. round bottomed heavy glass centrifuge tubes.
3. Blood of known hemoglobin content.

4. Accurately calibrated pipettes of 1, 2, 4, 5, 10, 15, and 20 cc. capacity.

5. Glacial acetic acid.

6. 50 cc. volumetric flasks.

7. 10 cc. volumetric flasks.

8. Purified benzidine reagent of Bing.

9 Test-tubes for the Evelyn photoelectric colorimeter.

10. 3 per cent hydrogen peroxide.

11. Paraffined corks to fit the colorimeter tubes.

12. No. 660 red filter for the colorimeter.

Procedure

The adequacy of the method as a whole was first tested on artificial mixtures containing known amounts of blood added to blood-free feces and a graph relating the quantity of blood to the colorimetric measurement was plotted (see Fig. 1). The operations necessary for obtaining the data for the curve are as follows:

1. Grind the fresh blood-free sheep feces in a mortar until the particles will pass through a screen having 10 meshes to the inch. Weigh thirty-five 1 gm. portions of the feces on an analytical balance and put each portion into a clean, dry, 50 cc. centrifuge tube.

2. Make a 1:10 dilution of a blood sample of known hemoglobin content with distilled water and mix thoroughly.

3. Number each centrifuge tube and carefully add the required amounts of diluted blood to each of the 1 gm. portions of feces, setting up the tubes in triplicate for each point on the curve to be determined. Mix the blood solution thoroughly with the feces, using individual glass stirring rods, and leave the rods in the tubes. Use the remaining two samples as duplicate blanks with blood omitted.

4. Add 10 cc. of distilled water to the blanks and proportionately less to the remaining tubes, so that the total volume of fluid added will be 10 cc. Mix thoroughly.

5. Add 15 cc. of glacial acetic acid to each tube and mix. Heat and remove each tube as soon as the contents begin to boil vigorously. Care must be taken to prevent the contents from boiling over. (The heating was facilitated by putting tubes in wire baskets and placing them on an electric hot-plate.)

6. Push particles adhering to the walls of the tubes back into

the solution, remove the stirring rods, and arrange them according to the number on the edge of a table with the acid-covered ends projecting so that none of the material on the rods may be lost. Centrifuge the tubes at approximately 2700 R.P.M. for 5 minutes.

7. Decant the supernatant fluid into properly numbered 50 cc. volumetric flasks. After transferring the contents of each tube, wash off the decanting rod with a small amount of glacial acetic acid.

8. Make three similar extractions, each with 10 cc. of glacial acetic acid, and bring the contents of the 50 cc. volumetric flasks up to volume with glacial acetic acid and mix thoroughly.

9. Put 1 cc. aliquots from the 50 cc. volumetric flasks into 10 cc. volumetric flasks and bring the contents of the 10 cc. volumetric flasks up to volume with glacial acetic acid and mix.

10. Put 2 cc. of the purified benzidine reagent into the bottom of each colorimeter tube and add 1 cc. aliquots from the 10 cc. volumetric flasks and mix.

11. Add 1 cc. of a 0.6 per cent hydrogen peroxide solution (3 per cent commercial solution freshly diluted with 4 parts of distilled water) and mix. Close with paraffined corks and allow the tubes to stand for 2 hours.

12. Dilute with 20 cc. of a 20 per cent solution of glacial acetic acid in distilled water, insert corks, and invert the tubes several times to mix the contents. Allow to stand for at least 8 minutes.

13. Adjust the colorimeter so that the blank reads 100 on the galvanometer scale when a No. 660 red filter is used. With this adjustment make the colorimetric readings on solutions derived from feces-blood mixtures and plot the average readings of triplicates against the volumes of blood employed.

Results

The results of a typical series of determinations made on sheep feces to which had been added the indicated quantities of calf blood containing 9 gm. of hemoglobin per 100 cc., together with the constant K_1 for each of the concentrations shown, are recorded in Table I.

The data in Table I show that the duplicate blanks checked perfectly, but that there was some variation in the readings obtained from the individual specimens making up each group of

triplicate samples containing a given amount of blood. In order to check further the reliability of galvanometer readings obtained from different samples of blood-free feces, a series of duplicate specimens from three healthy sheep on the same diet was tested at the same time and with the same reagents. The results of these tests are shown in Table II.

The data in Table II show that the standard deviation of the galvanometer readings obtained from the different samples of

TABLE I

Data from Which Curve Shown in Fig. 1 Was Plotted, together with Constant K_1 for Each Point Determined

Blood containing 9 gm. hemoglobin per 100 cc. added to 1 gm. sheep feces	Galvanometer readings*			Average galvanometer reading	Correction factor applied to average galvanometer readings	Corrected galvanometer readings	K_1
	A	B	C				
cc.							
0.00	100	100		100	0	100	
0.05	92	91^2	94	92^2	0	92^2	90.40
0.10	83	81	84	82^3	$+\frac{1}{4}$	83	107.87
0.20	62	63	66	63^3	$+\frac{1}{4}$	64	129.27
0.30	50	48	49	49	$+\frac{1}{4}$	49^2	135.56
0.40	41	38	37	38^3	$+\frac{3}{4}$	39^2	134.33
0.50	31	29	27	29	$+\frac{3}{4}$	29^2	141.33
0.60	20	22	21^2	21^2	$+\frac{3}{4}$	22	146.22
0.70	14^2	15^3	17	15^3	$+\frac{1}{2}$	16	151.62
0.80	11	11	10^2	10^3	$+\frac{1}{2}$	11	159.83
0.90	7	6^2	7^2	7	0	7	171.11
1.00	3	3^2	4	3^2	0	3^2	194.13
Average							141.97
Standard deviation...							±28.43

*Exponents refer to quarter units; *i.e.*, $91^2 = 91.50$.

blood-free feces was ±0.38, a variation which may be considered negligible in view of the extreme sensitivity of the colorimeter used. The differences in these readings, as well as those observed between samples within each set of triplicates, were apparently due to errors introduced during the numerous pipetting and diluting operations necessary to carrying out the procedure outlined, for they tended to decrease as the worker became more expert.

As is also shown in Table II, the readings obtained from each series of triplicate samples were averaged, and the average corrected as indicated in the correction table accompanying the galvanometer. The corrected galvanometer readings were then plotted against blood volume as illustrated in Fig. 1.

When the colorimeter readings are linearly proportional to the transmitted light intensity, and the colored solution obeys Beer's and Lambert's laws, the relation $(\alpha \log G)/C = K_1$ should apply, where G represents the galvanometer reading in per cent of that obtained from 100 per cent transmission, C represents the concentration, and K_1 is a constant. In the calculation of K_1 in

TABLE II

Galvanometer Readings Obtained from Different Samples of Blood-Free Sheep Feces

Sheep No.	Sample No.	Galvanometer reading
1	1	100.0
	2	99.5
2	1	100.0
	2	99.5
3	1	99.5
	2	99 0
Average		99.6
Standard deviation		±0.38
Average standard deviation		±0.15

Table I, C was expressed in mg. of hemoglobin in the colorimeter tube divided by 24, the volume of the colored solution in cc.

Although, as shown in Table I, the value of K_1 over the whole curve had a standard deviation of about twice the permitted variation of 10 per cent, from 0.2 to 0.9 cc., the variation was found to be only 9.68 per cent. This finding showed that between these points, and within the 10 per cent allowable variation, the curve followed the laws of Beer and Lambert, and indicated that along this portion of the curve the concentration of hemoglobin extracted from the feces was for all practical purposes proportional to the corrected galvanometer readings obtained from the colorimeter. Below 0.2 and above 0.9 cc., however, the curve deviated markedly from this straight line relationship, owing probably in the first instance to the impossibility of extracting

all of the hemoglobin, and in the second to the extreme concentration of the acid hematin with respect to the amount of benzidine reagent present.
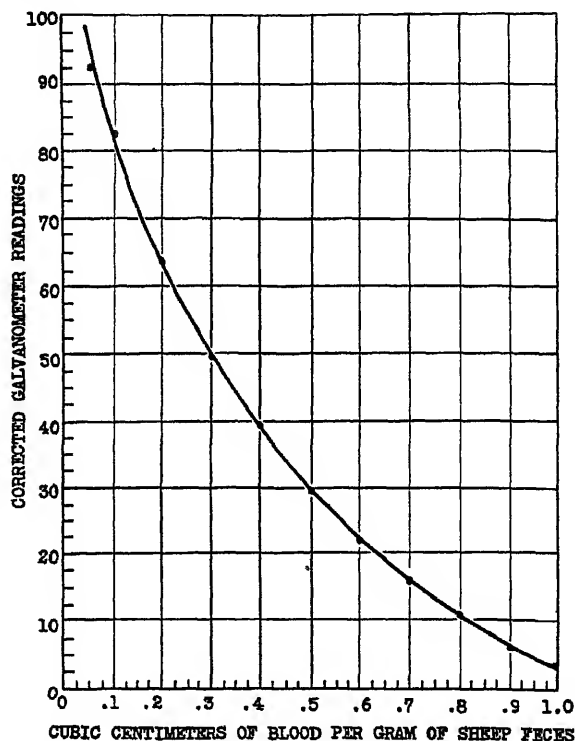
Fig. 1. Curve showing the corrected galvanometer readings obtained from the analysis of 1 gm. portions of sheep feces to which had been added the indicated volumes of calf blood containing 9 gm. of hemoglobin per 100 cc.

Any curve constructed on the basis of the foregoing procedure will be similar to the one illustrated, but will differ from it more or less on account of differences in the hemoglobin content of the blood sample and the purity of the benzidine reagent used.

Determination of Unknown Quantities of Blood in Sheep Feces

The procedure for determining the hemoglobin content of a sample of sheep feces containing an unknown quantity of blood

is the same as that just described with the following exceptions. (1) The total feces passed by the animal are weighed after screening; (2) additional blood is not added to the feces; (3) 10 cc. of distilled water are added to the blank and also to the feces containing the unknown quantity of blood; and (4) one blank and triplicate samples of the unknown are sufficient for the analysis.

As a practical test of the method, two sheep were drenched on several occasions with 200 to 400 cc. of blood from the same calf whose blood was used in plotting the curve in Fig. 1. The results of one of these tests following the administration of 400 cc. of blood mixed with 100 cc. of normal saline solution are recorded in Table III.

TABLE III

Results of Application of Present Method to Analysis of Sheep Feces Containing Unknown Quantities of Blood

Time after drenching	Weight of 24 hr. sample of feces	Average galvanometer readings	Blood indicated	Blood passed
hrs.	*gm.*		*cc.*	*cc.*
24	158	72	0.140	22.12
48	314	67	0.175	54.95
72	365	76	0.125	45.63
96	372	100	0.000	0.00
Total blood passed				122.70
Percentage of volume administered				30.70

The data in Table III show that the 30.70 per cent of the ingested blood which was recovered from the feces of this sheep was passed within 72 hours of the time of its administration. In four similar tests with sheep drenched with 200 cc. of blood, the 25 to 50 per cent of the total volume administered which was recovered was passed within 48 hours after ingestion. While these percentages varied considerably in the different tests and were also relatively small, it must be remembered that the blood did not originate within the alimentary canal of the animal in question, and also that, although an effort was made to insure its immediate entrance into the fourth stomach, it was not always certain that the blood went directly into that organ. Accordingly, losses caused by bacterial decomposition and possibly others occurring in the process of rumination could have taken place during the time the

blood remained in the rumen.   If, however, the blood had come from lesions within the digestive tract, as would have been the case in infections with stomach-worms or hookworms, it appears to the authors that a greater percentage of the blood lost by hemorrhage would be found in the feces, not only because the blood would enter the alimentary canal lower down and thus be subjected to less decomposition and absorption, but also because any stimulation of the intestine brought about by the presence of the infections would hasten its elimination.   While the authors can offer no proof for this statement, it appears to them to be a reasonable hypothesis and to make the method described of more value in studying the quantity of blood lost in such cases.

Since the curve in Fig. 1 is plotted in terms of cc. of blood having a known hemoglobin content, it is apparent that, if the concentration of hemoglobin in the blood of the animal used in the tests is known, the number of cc. of blood represented by the hemoglobin present in the feces may be expressed in terms of the volume of blood of the animal in question and this volume in turn may be related to any anemia that may be present.

DISCUSSION

In order that this method may be used successfully, five important points must be emphasized.

(a) The same lot of purified benzidine reagent should be used for plotting the reference curve and for making the determinations on unknown quantities of hemoglobin in any one experimental series.   In the course of the work leading up to the development of this method it was found that reference curves produced by different lots of purified benzidine were more or less parallel but were not identical.   This finding indicated that, although decolorization of any two samples of benzidine is carried out in exactly the same way, there may still be differences in the amounts of impurities in the compounds, which would make them useless unless employed in connection with their own reference curves.

(b) All determinations should be made on fresh feces.   It was found that when feces containing blood were allowed to remain at room temperature, or were dried at 50° and 100° for 24 hours, the amount of hemoglobin extracted was less than that obtainable from the same feces when fresh.   This adsorption and fixation

of the hemoglobin was particularly noticeable in those samples of feces dried at the higher temperatures.

(c) All weighing, pipetting, and diluting must be done with meticulous care. On account of the sensitivity of the color reaction between the benzidine reagent and the hemoglobin derivatives present in the acetic acid extract the slightest error in carrying out any of the operations indicated in the procedure will cause large variations in the resulting color and in the consequent galvanometer readings. It is, therefore, of utmost importance that these operations be performed with the greatest accuracy.

(d) Always use fresh glacial acetic acid extracts for making colorimetric comparisons. The acid hematin present in the acid extracts apparently precipitates out within a few hours in the acid solution, so that the color obtained from extracts 24 hours old is much weaker than that obtained from the same extracts when fresh. Accordingly, unless fresh extracts are used, the resulting color will not represent the original quantity of hemoglobin extracted from the sample.

(e) Do not use a blank as a reference point for any other similar sample containing blood unless the two tubes have been diluted at the same time. All of the diluted solutions in the colorimeter tubes, including the blank, tend to darken on standing. The color intensity of any one tube within a group, however, will read the same in the colorimeter until precipitation takes place about 24 hours after the tubes are set up, provided the machine is reset as the blank darkens.

It will be noted that the actual amount of acid hematin introduced into the colorimeter tubes in step (10) of the procedure represents only 1/500th of the total quantity of blood added to the feces in step (3), and the question might well be raised as to why the determinations were made on such a small portion of the original material. The reason for this technique lies in the fact that benzidine reacts to some extent with other pigments in the feces, and only by great dilution of the extracts could check blanks be obtained from the same lot of blood-free feces.

There is no reason why the method described in this paper may not also be applied to the quantitative determination of blood in the feces of other animals provided that (1) suitable blanks are available, (2) a satisfactory reference curve can be constructed,

and (3) interfering substances that may react with the benzidine reagent are absent.

The method for the quantitative determination of blood in sheep feces described in the present paper has been found to give satisfactory results in tests for the determination of the quantity of fresh blood (hemoglobin) added artificially to 1 gm. portions of blood-free sheep feces.

The recovery of 25 to 50 per cent of the total volume of blood ingested by healthy sheep from the feces of these animals by the use of this method indicated that it may be of some value in the study of gastrointestinal hemorrhage in sheep.

SUMMARY

A method for the quantitative determination of blood in the feces of sheep is described. The method was tested on the feces of sheep which had been drenched with measured volumes of calf blood and by its use 25 to 50 per cent of the ingested blood was recovered. All of the blood recovered was passed within 72 hours of the time of ingestion. The limitations of the method and its further application to the feces of other animals are discussed.

BIBLIOGRAPHY

1. van Eck, P. N., *Pharm. Weekbl.*, **61**, 1318 (1924).
2. Bing, F. C., and Baker, R. W., *J. Biol. Chem.*, **92**, 589 (1931).
3. Bing, F. C., *J. Biol. Chem.*, **95**, 387 (1932).
4. Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).

THE METABOLISM OF *l*-XYLULOSE*

By HARDY W. LARSON, N. R. BLATHERWICK, PHOEBE J.
BRADSHAW, MARY E. EWING, AND SUSAN D. SAWYER

*(From the Biochemical Laboratory of the Metropolitan Life Insurance
Company, New York)*

The exact nature of *l*-xylulose, one of the sugars occurring in
pentosuria, was definitely established by Levene and La Forge
(1) in 1914, but very little has been added to our knowledge of
this interesting substance since that time. We do not yet know
what rôle it plays in carbohydrate metabolism, or its source in
pentosuria. There is little evidence to substantiate the many
theories which have been advanced as to the probable cause of
xylulosuria and the anomaly remains one of the more obscure in-
born errors of metabolism. It has been suggested that xylulosuria
is inherited as a recessive factor (2) and that *d*-glucuronic acid is
a precursor (3) of the pentose. It may be pointed out also that
l-xylulose bears a close relationship to *l*-xylosone from which
ascorbic acid has been synthesized.

The sugar is usually excreted in small concentrations (less
than 1 per cent) at a fairly constant rate which appears to be
more or less independent of diet. About 100 cases of xylulosuria
have been reported in the literature to date. Since June, 1936,
we have examined for xylulose all urines coming to this laboratory
which showed a reduction of 0.25 per cent or more by Benedict
and Osterberg's picrate method (4). 89 cases of xylulosuria have
been found, making its incidence of occurrence one in about
50,000. It is realized that many instances of pentosuria may
have been missed in those cases showing less than 0.25 per cent
reducing material. The majority of the above cases had a pentose

---

* This paper was presented before the Thirty-second annual meeting of
the American Society of Biological Chemists at Baltimore, March 30 to
April 2, 1938.

excretion of 0.5 per cent or less. Judging from their names, all of the above persons having xylulosuria were Jews. One case was found in which pentosuria was associated with diabetes mellitus; this is believed to be the first recorded instance of such a combination. The urine of this person contained 2.19 per cent sugar of which 0.16 per cent was xylulose.

The present study was undertaken in order to secure information as to the metabolism of this sugar.

Preparation of l-Xylulose—Because *d*-xylose was readily procurable in sufficiently large quantities, we first synthesized *d*-xylulose, the optical isomer of the naturally occurring *l*-xylulose, and studied its metabolism (5). In order to prepare *l*-xylulose, it was first necessary to synthesize about 1.5 kilos of *l*-xylose, because the yield of ketose from xylose is very poor.

The *l*-xylose was prepared from *d*-sorbitol by the von Vargha (6) synthesis. The sorbitol was converted to monobenzal sorbitol, and then oxidized with lead tetraacetate (7). *l*-Xylose was obtained by hydrolyzing the resulting monobenzal *l*-xylofuranose

l-Xylulose was prepared by the method of Schmidt and Treiber (8). When *l*-xylose is refluxed for $4\frac{1}{2}$ hours with anhydrous pyridine, about 10 per cent is converted to *l*-xylulose and various other sugars. About 70 per cent of the unchanged *l*-xylose can be recovered by distilling off the pyridine *in vacuo* and dissolving the remaining crude syrup in absolute alcohol from which most of the xylose crystallizes. The addition of small amounts of absolute ether to turbidity facilitates the crystallization. After the removal of the xylose crystals, the ether and alcohol are distilled off *in vacuo*.

The separation of *l*-xylulose from the resulting mixture of crude syrups presents many difficulties. Our attempts to separate the xylulose from the acetone compounds of the mixed sugars by fractional distillation under a high vacuum were unsuccessful, and we had to resort to the use of *p*-bromophenylhydrazine to effect the separation, although this treatment gives extremely small yields. After treatment with norit, the crude syrup was evaporated to dryness and dissolved in a minimum amount of absolute alcohol. The mixture was brought to 70° and treated with slightly less than the calculated amount of recrystallized *p*-bromophenylhydrazine and placed in a vacuum desiccator over sulfuric acid

to crystallize.[1] Unless the sugar mixture contains a high percentage of xylulose and the *p*-bromophenylhydrazine is of the greatest purity, crystallization takes place very slowly and may require several weeks. For our experiments, Kahlbaum's purest *p*-bromophenylhydrazine was treated with norit in ether, recrystallized twice from ether, and used immediately after crystallization.

When the hydrazone crystals had set to a thick paste, they were transferred to a 50 cc. centrifuge tube, washed with a very small amount of ice water, and centrifuged. The brown liquid was then decanted and the washings repeated until no longer colored. After being washed with a small amount of ether, the xylulose *p*-bromophenylhydrazone was dissolved in a minimum amount of hot alcohol, and water was then added to turbidity. After the mixture had been allowed to stand in the refrigerator an hour, the crystals were centrifuged off. The several washings and the recrystallization of the hydrazone result in great loss, but are necessary in order to secure a pure sugar.

Xylulose was liberated from the hydrazone by treatment with benzaldehyde. The hydrazone crystals were dissolved in water on the steam bath, and the calculated amount of benzaldehyde added with vigorous stirring. Heating and stirring were continued on the steam bath for $1\frac{1}{2}$ hours and a few drops of benzaldehyde were added from time to time to insure an excess. The filtrate was washed seven times with 100 cc. portions of ether. Then the solution was treated with a very small amount of norit, filtered, and the water distilled off *in vacuo* at 40°. The *l*-xylulose thus obtained gave a rotation of $+33.4° \pm 0.2°$.

<div style="text-align:center">EXPERIMENTAL</div>

The xylulose experiments were made on white rats of a uniform strain (aged 87 to 118 days) which had been fed the same stock diet. The animals were subjected to a preliminary fast of 24 hours after which 2 cc. of a 25 per cent xylulose solution were administered by stomach tube. 3 hours after the ingestion of the sugar the animals were anesthetized with sodium amytal and

[1] Dr I Greenwald generously furnished us seeding crystals of the hydrazone

TABLE I

Carbohydrate Content of Tissues of Control and Experimental Rats

The results are expressed in mg. per 100 gm., except for blood, in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose. The figures in bold-faced type represent values for the rats given *l*-xylulose.

Substance determined	Maximal	Minimal	Mean	Standard deviation of the mean	No. of rats Males	Females
Glycogen, liver	603	26	203	28		30
" " ..	593	125	306	53		9
" "	1847	94	535	67	41	
" " ...	704	104	376	73	9	
" muscle.	749	370	563	11	40	29
" " .	588	390	486*	13	9	9
Lactic acid, liver .. .	17.5	5.5	10.0	0.5	23	14
" " " . ..	20.6	6.4	9.4	0.8	9	9
" " muscle ..	54.2	11.8	22.8	1.2	31	20
" " "	37.9	8.6	21.7	2.0	9	9
" " blood..	21.8	8.1	13 8	1.0	8	8
" " "	29.9	9.6	16.3	3.5	3	2
Fermentable reducing substances						
Liver	220	51	104	4.1	34	19
"	164	48	105	2.3	9	9
Muscle.............	42	4	17	1.0	36	19
"	30	4	17	1.7	9	9
Kidney... . . .	82	15	53	2.0	35	19
"	109	27	59	5.5	9	9
Blood	92	59	74	1.7	11	12
"	105	77	85*	5.3	3	2
Non-fermentable reducing substances						
Liver	59	0	26	1.9	34	19
"	99	11	42*	1.8	9	9
Muscle.....	45	4	17	1.2	36	19
"	32	9	20	1.6	9	9
Kidney....	37	5	17	1.1	35	19
"	87	8	30*	4.4	9	9
Blood	8	2	5	0.4	11	12
"	10	5	8*	0.9	3	2

* Statistically significant by the *t* test of Fisher.

as soon as anesthesia was complete the desired tissues were frozen *in situ* and then transferred to liquid air. The procedure followed and the methods used for analysis of the various tissues have been described by Blatherwick *et al.* (9). Glycogen, lactic acid, and fermentable and non-fermentable reducing substances of liver and muscle were determined; also lactic acid and fermentable and non-fermentable reducing substances of the kidney. The values for the control rats are those obtained over a period of 4 years in separate experiments dealing with carbohydrate metabolism. The observations were sufficiently uniform to justify combining them. As the liver glycogen of fasted males is greater than that of females, we have separated the liver glycogen values according to the sex of the animal. The maximal, minimal, and mean values are given together with the standard deviation of the mean. The means which are statistically significant by the *t* test of Fisher (10) are designated.

From an inspection of the results shown in Table I there is little evidence to indicate that *l*-xylulose is metabolized in the rat. No significant change from control values occurred in the liver glycogen or in the lactic acid content of muscle, liver, and blood. Neither was there any significant change in the fermentable reducing substances of muscle, liver, and kidney. There was, however, a significant decrease in the glycogen of muscle comparable to that reported after the feeding of *l*-xylose (7) and an increase in the fermentable sugar of the blood which was significant. Similar increases in blood glucose have been noted after the administration of *d*-xylulose (5) and *d*-xylose (11). Since in the present study only five rats were used for the determination of blood sugar, too much importance should not be placed on these results. Non-fermentable reducing substances of the liver, kidney, and blood showed significant increases indicating absorption of the sugar. Only a trace of the xylulose given appeared in the urine.

<div style="text-align:center">DISCUSSION</div>

The experiments show that *l*-xylulose, the naturally occurring sugar, is not metabolized in the rat. This result was not expected on the basis of earlier experiments showing that *d*-xylulose, which

is not found in the body, is utilized (5). The administration of *d*-xylulose to rats resulted in a 6-fold increase in the liver glycogen of females and in a 2-fold increase in the case of males. It seems probable that there is a species difference in the metabolism of *l*-xylulose, since the depancreatized dog transforms some of this sugar to glucose (12). The diabetic dog excreted from 92 to 97 per cent of the ingested and injected sugar of which only approximately one-third was non-fermentable.

The possibility must be considered that the failure of *l*-xylulose to be metabolized in the rat may be due to its low rate of absorption from the gastrointestinal tract. The coefficient of absorption of this sugar is only 0.044 as compared with 0.131 for *d*-xylulose and 0.074 for *d*-xylose. These coefficients are averages of results for both females and males. The rate of absorption of *l*-xylulose for females is 0.056 and 0.028 for males. Glycogen formation resulted when glucose was given in the quantity equivalent to the *l*-xylulose absorbed. The liver glycogen content of a group of nine female rats averaged 472 mg. per 100 gm. in comparison with the control value of 203 mg., while the corresponding values for six males were 752 and 535 mg.

According to Verzár and McDougall (13) pentoses are absorbed from the intestine by simple diffusion which is dependent on concentration and are not selectively absorbed as the hexoses arc, because they do not undergo phosphorylation in the intestine. In accordance with this theory *d* and *l* forms of aldo- and keto-pentose should be absorbed at the same rate and therefore have the same coefficient. However, the coefficients of absorption which we have found for *d*- and *l*-xylose and *d*- and *l*-xylulose are 0.074, 0.007, 0.131, and 0.044 respectively. It is apparent, therefore, from these figures that the configuration of the substance in question is a factor influencing its diffusion or selective absorption.

The selective permeability of the gastrointestinal mucosa for these sugars of identical configuration is indeed strange, as is the utilization of one ketose and its ability to form glycogen and the apparent failure of its optical isomer to be metabolized. Hamburger (14) has shown a similar and even more remarkable selectivity in the kidney for slight structural variations of a single isomer. He found that the glomerular membrane retained the

α form of l-xylose but on the other hand permitted the passage of the β form of the same sugar. The recent work of Rudney (15) on the metabolism of l-glucose is analogous to our findings. He showed that this sugar was not metabolized by the rat, approximately 85 per cent of the l-glucose being excreted within 24 hours after intravenous injection. l-Glucose was neither oxidized nor fermented by surviving tissue slices of rat brain or Sarcoma 39 nor did it affect the oxidation of d-glucose by these tissues. Rudney demonstrated that *Escherichia coli communis* and *Aerobacter aerogenes* grew on d-glucose but not on l-glucose. Acid and gas were formed in 24 hours by both bacteria in the presence of d-glucose but not in the presence of l-glucose. The work of Cori and Cori (16) on the metabolism of d- and l-lactic acid is well known. They were able to show that d-lactic acid was utilized by the rat to form liver glycogen, whereas very little glycogen was produced from the l form. Further evidence of this difference in the utilization of optical isomers has been well established in the case of the amino acids.

SUMMARY

l-Xylulose is not metabolized by the rat. No significant rise in the glycogen content of liver and muscle nor in the lactic acid content of liver, muscle, and blood was observed after its administration. Non-fermentable reducing substances in the liver, muscle, kidney, and blood were increased. A significant increase in the fermentable sugar of the blood occurred.

BIBLIOGRAPHY

1. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **18**, 319 (1914).
2. Lasker, M., Enklewitz, M., and Lasker, G. W., *Human Biol.*, **8**, 243 (1936).
3. Enklewitz, M., and Lasker, M., *J. Biol. Chem.*, **110**, 443 (1935).
4. Benedict, S. R., and Osterberg, E., *J. Biol Chem.*, **48**, 51 (1921).
5. Larson, H. W., Blatherwick, N. R, Bradshaw, P. J., Ewing, M. E., and Sawyer, S. D., *J. Biol. Chem.*, **117**, 719 (1937).
6. von Vargha, L., *Ber. chem. Ges.*, **68 B**, 18 (1935).
7. Larson, H. W., Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., and Sawyer, S. D., *J. Biol. Chem.*, **136**, 1 (1940).
8. Schmidt, O. T., and Treiber, R., *Ber. chem. Ges*, **66 B**, 1765 (1933).

9. Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., Larson, H. W., and Sawyer, S. D., *J. Biol. Chem.*, **111**, 537 (1935).
10. Fisher, R. A., Statistical methods for research workers, London and Edinburgh, 6th edition (1936).
11. Blatherwick, N. R., Bradshaw, P. J., Cullimore, O. S., Ewing, M. E., Larson, H. W., and Sawyer, S. D., *J. Biol. Chem.*, **113**, 405 (1936).
12. Larson, H. W., Chambers, W. H., Blatherwick, N. R., Ewing, M. E., and Sawyer, S. D., *J Biol. Chem.*, **129**, 701 (1939).
13. Verzár, F., and McDougall, E. J., Absorption from the intestine, London, New York, and Toronto (1936).
14. Hamburger, H. J., *Biochem. Z.*, **128**, 185 (1922).
15. Rudney, H., *Science*, **92**, 112 (1940).
16. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **81**, 389 (1929).

BLOOD URIC ACID AND LIVER URICASE OF ZINC-DEFICIENT RATS ON VARIOUS DIETS*

By L. W. WACHTEL, E. HOVE, C. A. ELVEHJEM, and E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

The necessity of zinc in rat nutrition was first shown conclusively by Todd, Elvehjem, and Hart (1). In this and in subsequent work a high carbohydrate, zinc-low ration has been used. The possibility exists that the zinc requirement of the rat may be influenced by rations in which fat or protein furnishes the chief source of calories.

A physiological relationship between zinc and vitamin B_1 has been suggested by certain reports in the literature. Lohmann and Kossel (2) have shown that zinc ions activate the enzymatic decarboxylation of pyruvic acid when a yeast maceration juice is the source of the protein portion of the carboxylase system. Eggleton (3) found a 50 per cent decrease in the zinc content of skin, hair, and nails of persons suffering from beriberi. Both Eggleton (3) and Morris (4) have noted a correlation between the zinc and vitamin B_1 content of foods. Since both zinc and vitamin B_1 are required by the rat on the usual high carbohydrate diet, it is possible that any interdependence which may exist between these two nutrients can be demonstrated by placing rats on a high fat, zinc-low diet. If such a diet has a zinc-sparing action as well as its known vitamin B_1-sparing action, an interdependence between these two substances may be indicated. If there is no zinc-sparing action of the high fat diet, it can be con-

cluded that only a small part, if any, of the zinc requirement *in vivo* is involved directly in cocarboxylase function. Our results on such an experiment are reported in this paper.

Previous work from this laboratory (5–7) has shown an abnormal metabolism of nitrogen in the zinc-deficient animal; several factors have indicated a preferential delay in the absorption of nitrogenous compounds from the intestinal tract. Such a condition of partial protein starvation may be expected to produce an accelerated catabolism of body proteins, leading to an increase in the level of uric acid in the blood of the zinc-deficient rat. As expected, a marked increase in this constituent of the blood has been found. On the basis of these results it seemed important to investigate the effect of a high protein, zinc-low ration on the uric acid level of the blood. We have also placed rats on a high purine ration, expecting to see a more severe deficiency. The results of these experiments are reported in this paper.

In connection with the uric acid studies it is interesting to note that Holmberg (8) has shown a highly purified uricase preparation to contain 0.13 per cent zinc. Therefore, we have made uricase determinations on the livers of zinc-deficient animals.

<center>EXPERIMENTAL</center>

The compositions of the rations used in these experiments are indicated in Table I. The purifications of the constituents and the preparations of the animals are the same as outlined in a previous paper (6). The lard used in the high fat ration was purified by washing several times with 1 per cent HCl followed by distilled water. The estimated daily zinc intakes on these rations are also given in Table I. The rats in the control group received 100 γ of zinc per day as the sulfate.

The growth records are given in Table II which is divided into two parts. Part I contains the growth data on the high fat as compared with the high carbohydrate ration, and Part II contains the data on the high protein and high purine as compared with the high carbohydrate ration. It appears evident that the type of diet had little if any effect on the severity of the zinc deficiency or the rate at which the rats showed evidence of such deficiency.

At the conclusion of the 6 week growth period uric acid was determined in the blood plasma of all rats by the method of Folin

and Svedberg (9).   The results of these determinations are shown in Table II.   It is evident that the uric acid of the zinc-deficient rats is considerably higher than for the control animals receiving zinc, and that the difference is not affected by the type of diet.

Four of the zinc-deficient rats on the carbohydrate diet were placed on the zinc supplement at this time.   In three of the rats the growth response during the 1st week was phenomenal, being over 60 gm. in one case.   However, in spite of this remarkable

TABLE I

Composition of Zinc-Low Rations

	High car-bohydrate	High fat	High protein	High purine
Egg white, %.... . . .	14 0	22 0	60.0	14.0
Sucrose, %	79.0	15.0	33.0	78.0
Lard, % .. .		55 0		
Corn oil, %.. .	2 0		2.0	2.0
Salts 45T (1), % . . .	4 0	6.0	4.0	4.0
Liver extract,* %... . .	1.0	1 6	1.0	1.0
Choline, %		0.5		
Vitamin B_1,† γ *per gm*.... . ..	1	2	1	1
" B_6,† γ " " . .	3	6	3	3
Uric acid, %				1.0
Average daily food consumption,				
gm.	5 5	4.3	6.5	6.3
Zinc in ration, γ *per gm*....	1.0	1.4	1.8	1.1
Average daily zinc ingestion, γ	5.5	6.0	11.7	7.0

* Purified by extraction of a water solution with dithizone in CCl_4.

† Vitamins B_1 and B_6 were kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

growth response to zinc the blood uric acid remained as high as before.   In the following weeks the blood uric acid showed a gradual decline until at the end of 5 weeks on the zinc supplement all rats but one had uric acid values which approximated the normal.

Creatinine was determined in the whole blood of five rats receiving zinc and six zinc-deficient rats by the standard picric acid method.   No differences were observed.   The range for the rats receiving zinc was 4.5 to 6.6 mg. per 100 cc. of blood with an average of 5.3; for the deficient animals the range was 4.9 to 6.6

TABLE II

Growth Records and Plasma Uric Acids of Rats on Various Zinc-Low Diets

Ration	Added zinc				Zinc-low			
	Sex	Initial weight	Average weight gain per day	Uric acid per 100 cc. plasma	Sex	Initial weight	Average weight gain per day	Uric acid per 100 cc. plasma
I. Growth for 6 wks. on high fat as compared with high carbohydrate ration								
		gm.	*gm.*	*mg.*		*gm.*	*gm.*	*mg.*
Carbohydrate	♀	28	2.62	2.72	♀	33	1.43	6.19
	♂	28	3.38	3.69	♂	31	1.55	5.71
	♀	44	2.79	4.45	♀	47	1.31	
	♂	29	4.34	3.30	♂	32	1.84	4.77
	♀	26	2.79	3.84	♀	25	0.95	6.48
					♀	28	1.60	5.55
Average	♂		3 86	3.60	♂		1.70	5.74
"	♀		2.73		♀		1.32	
Fat	♀	32	2.74	4.35	♀	26	1.98	7.64
	♂	25	2.95	4.53	♀	27	1.19	6.68
	♂	39	2.41	2.89	♂	39	1.33	5.25
	♂	34	3.88	3.18	♂	35	2.53	5.83
	♀	33	2.93	4.63	♂	33	1.95	6.36
	♂	21	2.83	3.94	♂	24	1.84	4.99
Average. ...	♂		3.02	3.92	♂		1.91	6.13
"	♀		2.84		♀		1.59	
II. Growth for 4 wks. on high protein and high purine as compared with high carbohydrate ration								
Carbohydrate	♀	53	2.32	3.55	♂	52	0.61	7.56
	♂	57	2.68	4.29	♀	46	0.64	7.41
	♂	70	3.50	3.49	♂	49	0.61	6.61
Average.......	♂		3.09	3.89	♂		0.61	7.09
Purine	♂	58	2.60	6 39	♂	56	1.21	7.78
	♂	58	2.75	5.48	♂	51	0.82	6.81
	♂	75	3.50	4.32	♂	75	0.54	7.05
Average.......			2.95	5.40			0.86	7.21
Protein	♀	49	2.54	3.76	♂	52	1.07	
	♂	40	4.07	4.99	♀	59	0.68	7.73
	♂	65	3.46	3.19	♀	51	0.86	6 84
					♂	61	0.86	5.06
					♀	67	1.11	5.92
Average.......	♂		3.77	4.09	♂		0.97	6.39
"					♀		0.88	

mg. per 100 cc. with an average of 5.5 mg. per 100 cc. A few determinations of the non-protein nitrogen of the blood were made on the rats receiving zinc and on the rats receiving a zinc-deficient diet but no significant differences were observed. In a previous paper (5) the results of the analysis for blood non-protein nitrogen of fifteen rats on a zinc-low and a zinc-supplemented ration showed an average decrease of 14 per cent in the zinc-deficient animals although there was some overlapping of the ranges.

Uricase Determinations—Livers of freshly killed rats were removed, minced, weighed, and washed three times with 0.9 per

TABLE III

Liver Uricase of Rats on Zinc-Deficient and Zinc-Supplemented Rations

Ration	Added zinc		Zinc-low	
	No. of rats and sex	Uricase	No. of rats and sex	Uricase
		c.mm. O₂ per min. per mg.		*c.mm. O₂ per min. per mg.*
Carbohydrate	4 ♂	0.081	4 ♂	0.078
	4 ♀	0.095	4 ♀	0.089
Fat	4 ♂	0.067	4 ♂	0.074
	2 ♀	0.088	2 ♀	0.080
Protein	2 ♂	0.097	1 ♂	0.098
	1 ♀	0.104	3 ♀	0.093
Purine	2 ♂	0.061	1 ♂	0.083
Grand average...	12 ♂	0.076	10 ♂	0.079
" "	7 ♀	0.094	9 ♀	0.088

cent saline, being centrifuged between washings. The minced tissue was taken up in enough 0.05 M borate buffer, pH 9.0, to give a final suspension of 200 mg. of fresh tissue per cc. This was then homogenized according to the technique of Potter and Elvehjem (10). Dry weight determinations were made on these homogenized suspensions. In the determination of uricase 1 cc. of the tissue suspension and 1.5 cc. of borate buffer were placed in the Barcroft flask and 0.5 cc. of the substrate solution, containing 10 mg. of uric acid as the lithium salt, was introduced from a dangling cup. The reaction was allowed to proceed at 39°, and the oxygen uptake measured at 10 minute intervals for 30 minutes.

The results are expressed as the oxygen uptake per minute per mg. of dry weight of tissue. The values obtained on the rats receiving a zinc-deficient ration and one supplemented with zinc are shown in Table III. No differences were observed.

In contrast to liver suspensions, kidney suspensions from normal rats were found to be practically devoid of uricase activity.

Some Properties of Uricase—Since Holmberg (8) has obtained evidence that zinc is a constituent of the uricase molecule, an attempt was made to demonstrate an inhibition of this enzyme by potassium thiocyanate. This compound is known to be a strong inhibitor of carbonic anhydrase, which is also a zinc-containing

TABLE IV

Partial Purification of Hog Liver Uricase*

	Zinc content in dry matter	Specific activity
	per cent	c.mm. O_2 per min. per mg.
Fresh, minced hog liver. .	0.049	0.054
Acetone-dried " " . . .	0 020	0.070
Borate buffer (pH 10) extract; cleared with barium acetate	0.053	0.34
$(NH_4)_2SO_4$ ppt.; heat denaturation; dialysis for 28 hrs..	0.083	9.40

* The procedure of Davidson (12) has been followed.

enzyme (11). However, thiocyanate had no effect on uricase, even at such a high concentration as 22 mM. In contrast, cyanide inhibited completely at a 1 mM concentration. Inorganic zinc (as sulfate) had no effect at a level of 0.1 mM final concentration; at 0.5 mM zinc a 10 per cent inhibition resulted, and at 2.0 mM an 80 per cent inhibition was produced. The effects of zinc and thiocyanate were tried both on a crude rat liver suspension and on a purified uricase solution prepared from pork liver.

The purified pork liver uricase used in the above studies was prepared as indicated in Table IV. The activity has been increased nearly 200 times, while the zinc content has increased as yet only 4 times. Although there does appear to be a steady increase in the zinc content, no conclusion as to the presence of zinc in the uricase molecule can be made until more intensive purification studies are carried out.

Since the high fat ration produced no apparent reduction in the zinc requirement of the rat, it can be tentatively concluded that only a small amount, if any, of the daily zinc ingested is involved directly in cocarboxylase function; this conclusion is dependent on the assumption that the marked decrease in the dietary vitamin B_1 requirement on high fat diets represents a true physiological decrease in the vitamin B_1 requirement, and there is some evidence for this assumption (13).

The zinc deficiency produced on the high protein ration was of about equal severity and showed about the same rate of onset as that produced on the high carbohydrate diet.   Furthermore, the abnormally high blood uric acid was fully as prominent in zinc-deficient rats on the high protein as on the high carbohydrate diet. From this it appears that the high uric acid is not the result of protein starvation in the zinc-deficient animal with subsequent breakdown and utilization of body tissues.   This point is supported also by the fact that the blood uric acid does not return to normal until fully 5 weeks after a zinc-deficient rat is placed on a ration with zinc added.   Apparently the body mechanism for the production of uric acid has undergone some deep seated derangement during the course of the zinc deficiency.   Other nitrogen compounds in the blood of zinc-deficient animals appear to be relatively normal.   Creatinine is normal and the blood non-protein nitrogen is normal or at most shows only a slight fall; therefore, by difference, blood urea ought to be slightly below normal.   It may be worth mentioning that this is the same blood nitrogen picture as is produced by injections of small amounts of insulin in normal animals (14).

The zinc deficiency produced on the ration containing added uric acid was no more severe than on the same ration without the uric acid.   This seems to indicate that the high blood uric acid in the zinc-deficient animals is due, not to an inability of the rat to eliminate or break down uric acid formed in the body, but rather to an excessive rate of production of uric acid by the organism. Supporting this is the fact that the zinc-deficient animal has an entirely normal concentration of uricase in its liver.

The fact that we have been unable to demonstrate a decrease in liver uricase in the zinc-deficient animal does not necessarily

contradict the reported presence of zinc in the uricase molecule (8). An analogous situation was seen in the case of carbonic anhydrase; this red blood cell enzyme has been definitely shown to contain zinc; yet no significant decrease in concentration in the blood of zinc-deficient rats was observed (11).

SUMMARY

1. High fat diets have been found to exert no zinc-sparing action. This is discussed with respect to a postulated interdependence of zinc and vitamin B_1.

2. Neither high protein nor high purine diets have an effect on the severity or rate of onset of the zinc deficiency in rats.

3. A marked and persistent rise in plasma uric acid of zinc-deficient rats on all rations has been observed. When zinc-deficient rats were placed on the control ration with zinc added, the blood uric acid returned to normal only after several weeks, although a pronounced growth stimulation resulted immediately.

4. The concentration of uricase in the livers of the zinc-deficient rats was normal. This fact, along with the effect of certain inhibitors on this enzyme, is discussed in terms of the reported presence of zinc in the uricase molecule.

BIBLIOGRAPHY

1. Todd, W. R., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, **107**, 146 (1934).
2. Lohmann, K., and Kossel, A. J., *Naturwissenschaften*, **35**, 595 (1939).
3. Eggleton, W. G., *Biochem. J.*, **33**, 403 (1939).
4. Morris, J. P., *Brit. Med. Assn., J. Malay Branch*, **4**, 114 (1940).
5. Hove, E., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, **119**, 768 (1937).
6. Hove, E., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, **124**, 750 (1938).
7. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **134**, 425 (1940).
8. Holmberg, C. G., *Biochem. J.*, **33**, 1901 (1939).
9. Folin, O., and Svedberg, A., *J. Biol. Chem.*, **88**, 85 (1930).
10. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
11. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **136**, 425 (1940).
12. Davidson, J. N., *Biochem. J.*, **32**, 1386 (1938).
13. Stirn, F. E., Arnold, A., and Elvehjem, C. A., *J. Nutrition*, **17**, 485 (1939).
14. Jensen, H. F., Insulin, London, 184 (1938).

ACETYLATION IN VIVO OF p-BROMOPHENYL-d-CYSTEINE

By VINCENT du VIGNEAUD, JOHN L. WOOD, and
FRANCIS BINKLEY

*(From the Department of Biochemistry, Cornell University Medical College,
New York City)*

During the studies of the inversion of d-amino acids to their antipodes within the animal body it has been established that d-phenylaminobutyric acid can be converted to and excreted as the acetyl derivative of l-phenylaminobutyric acid (1). The d-amino acid was regarded as being converted by deamination to the keto acid with the keto acid being converted to the amino acid by the pyruvic acid acetylating mechanism (1). By the use of the N^{15} isotope it was possible to demonstrate that the original nitrogen of the d-phenylaminobutyric acid was in great measure lost at some stage in the inversion (2). On the other hand, it was found that when l-phenylaminobutyric acid was fed, the acetyl derivative which was excreted retained practically all of the original nitrogen. The various possibilities of explaining these sets of facts were considered in much detail in the previous presentation (2). Among several possibilities, it was recognized that direct acetylation of the l-amino acid was one of the possible explanations of the retention of the original nitrogen in the formation of acetyl-l-phenylaminobutyric acid from l-phenylaminobutyric acid. The possibility of direct acetylation of the d-phenylaminobutyric acid did not come into question, since no indication of acetyl-d-phenylaminobutyric acid formation was obtained.

A hint of the possibility of direct acetylation of an amino acid of the d configuration was obtained in the extension of these studies to the conversion of S-benzyl-d-cysteine to N-acetyl-S-benzyl-l-cysteine (3). As we had reported, a small amount of the dl compound was isolated from the urine each time the benzyl-d-

369

cysteine was fed, although the preponderant product was the acetylbenzyl-*l*-cysteine. The amounts of *dl* compound isolated were not large enough to remove all doubts as to whether its presence was due to racemization in the process of isolation. However, no evidence of racemization could be found in appropriate control experiments.

We now report an unequivocal case in which the *d*-amino acid is acetylated. After the feeding of *p*-bromophenyl-*d*-cysteine having 90 per cent of maximum rotation, an acetyl derivative having a rotation of +4° was isolated from the urine, whereas after a similar feeding of its enantiomorph, *p*-bromophenyl-*l*-cysteine of maximum rotation, the isolated acetyl derivative possessed a rotation of −13°. The rotation of acetyl-*p*-bromophenyl-*l*-cysteine obtained from *p*-bromophenyl-*l*-cysteine by acetylation with acetic anhydride was −13° under the same conditions. The acetyl derivative isolated after the feeding of the *d* isomer was thus predominantly the acetyl derivative of the *d* isomer. After the intraperitoneal injection of the same *p*-bromophenyl-*d*-cysteine the acetyl derivative isolated from the urine possessed a rotation of +11°, indicating less inversion with increased rate of administration.[1]

The relationship of these findings to the theories of acetylation of amino acids is being further investigated. The direct acetylation of the *d* isomer naturally focuses attention on the possibility of direct acetylation of the *l* isomer. The mechanism involved in the inversion and acetylation, where partial inversion of the *p*-bromophenyl-*d*-cysteine was obtained, can be explained on the

---

[1] As a possible explanation of the difference in the behavior between *d*-phenylaminobutyric acid and *p*-bromophenyl-*d*-cysteine it occurred to us that acetylation of the *d*-amino acid might take place if it were slowly deaminized. In a preliminary investigation in association with Dr. Fritz Lipmann, we have found that *p*-bromophenyl-*d*-cysteine is, in fact, much more slowly deaminized by *d*-amino acid oxidase than *d*-phenylaminobutyric acid. We therefore suggest that the *d*-amino acid which is not readily deaminized may be subject to the acetylation mechanism, whereas a *d*-amino acid like *d*-phenylaminobutyric acid which is readily deaminized is converted to the keto acid at such a rate that little, if any, direct acetylation of the *d*-amino acid occurs. The difference would therefore be quantitative rather than qualitative as far as acetylation is concerned. We hope to explore this possibility further.

basis of the same mechanism as that suggested for the inversions already presented (1–3).

Preparation of Optical Isomers of p-Bromophenylcysteine—The synthesis of p-bromophenyl-l-cysteine was carried out by a modification of the method of Friedmann (4). 10 gm. of l-cystine were dissolved in 200 cc. of 1.5 N H_2SO_4. 6 gm. of zinc dust were added and the mixture was heated on a water bath for 4 hours with the occasional addition of small pieces of mossy zinc. Cuprous oxide in aqueous suspension was added to the warm solution until the supernatant solution became blue and no more cuprous oxide went into solution.[2] A sulfuric acid solution of diazotized p-bromoaniline (18 gm.) was added dropwise with stirring to the ice-cold solution of cysteine cuprous mercaptide. A vigorous evolution of nitrogen took place. The stirring was continued for 1 hour and the solution was allowed to come to room temperature. After the solution had been heated for $\frac{1}{2}$ hour at 70°, it was cooled and was extracted with two 100 cc. portions of ethyl ether which were discarded. The aqueous solution was saturated with H_2S. The precipitate was removed by filtration and was washed with two 25 cc. portions of 1.5 N H_2SO_4. After the solution had been boiled to remove the excess H_2S, it was cooled and was neutralized slowly to Congo red by the dropwise addition of concentrated NH_4OH. 7.2 gm. of p-bromophenyl-l-cysteine were obtained. This represented 46 per cent of the theoretical yield of p-bromophenyl-l-cysteine, allowance being made for 3.1 gm. of l-cystine recovered from the mother liquors. The p-bromophenyl-l-cysteine possessed a melting point of 193° and a specific rotation of $[\alpha]_D^{22} = +18°$ for a 1 per cent solution in 0.1 N NaOH. The compound possessed 39.35 per cent C and 3.70 per cent H in close agreement with the theoretical values of 39.14 and 3.65 respectively.

The compound was found to be identical with p-bromophenyl-l-cysteine prepared from l-mercapturic acid isolated from the urine of a dog fed bromobenzene.

The p-bromophenyl-d-cysteine was prepared from d-cystine

---

[2] We wish to acknowledge the suggestion of Professor Hans Clarke that we use the cuprous mercaptide for the synthesis.

in the same manner as that used for the enantiomorphic form. The *d*-cystine used in the synthesis was prepared by the racemization and resolution procedure of Wood and du Vigneaud (5).

Preparation of Acetyl-p-Bromophenyl-l-Cysteine—500 mg. of the *p*-bromophenyl-*l*-cysteine were dissolved in 2 cc. of 1 N NaOH and 0.5 cc. of acetic anhydride was added in five portions, each of which was preceded by 1 cc. of 2 N NaOH. The mixture was immersed in an ice bath and stirred vigorously during the additions. 15 minutes after all the anhydride had been added, an amount of H_2SO_4 was added equivalent to the total amount of NaOH which had been employed in the acetylation. The precipitated material was washed with water and was dried. The dried material was dissolved in acetone, filtered, and precipitated by pouring into warm water. 300 mg. of acetyl derivative melting at 152° and having a rotation of $[\alpha]_D^{23} = -13°$ were obtained. It was identical in properties with acetyl-*p*-bromophenyl-*l*-cysteine isolated from the urine of a dog receiving bromobenzene. The latter melted at 152–153° and possessed a rotation of $[\alpha]_D^{25} = -13°$ and showed no depression in the melting point when mixed with an equal amount of the synthetic compound.

Feeding Experiments

p-Bromophenyl-l-Cysteine—2 gm. of *p*-bromophenyl-*l*-cysteine, having a rotation of $[\alpha]_D^{22} = +18°$, were mixed with 30 gm. of stock diet and the mixture was given *ad libitum* to a 290 gm. male white rat. The food was eaten within 7 days and the urine was collected for 9 days. The collecting funnel was washed with dilute NH_4OH and the washings were combined with the urine. After filtration through glass wool the urine was made alkaline to litmus and was extracted once with an equal volume of chloroform. The urine was then acidified to Congo red by the addition of HCl and was extracted three times with equal volumes of chloroform. The combined chloroform layers from the extraction of the acidified urine were centrifuged to break the emulsion which formed. After the solution was dried with anhydrous Na_2SO_4, the chloroform was removed by distillation and the residue was dissolved in dilute NH_4OH. The solution was treated with a small amount of Darco, filtered, and acidified to Congo red by the addition of HCl. The resulting crude precipi-

tate weighed 700 mg. Recrystallization of the material gave 620 mg. of acetyl-*p*-bromophenyl-*l*-cysteine with a melting point of 152° and a rotation of $[\alpha]_D^{27} = -13°$ for a 1 per cent solution in 95 per cent ethanol. The analytical value found for nitrogen was 4.33 as compared with the theoretical value of 4.40.

The feeding of *p*-bromophenyl-*l*-cysteine was repeated several times with practically identical results.

p-Bromophenyl-d-Cysteine—0.5 gm. of *p*-bromophenyl-*d*-cysteine having a rotation of $[\alpha]_D^{27} = -13.5°$ was mixed with 10 gm. of Bal-Ra dog chow and fed to two rats over an 8 day period. 115 mg. of crystalline acetyl derivative were isolated from the collected urine. Upon purification the derivative melted at 153° and possessed a rotation of $[\alpha]_D^{27} = +5.5°$.

In another experiment a rat was fasted 24 hours and was given 0.4 gm. of bromophenyl-*d*-cysteine of $[\alpha]_D^{27} = -13.5°$ in 2 gm. of Bal-Ra dog chow. The animal ate this immediately but then refused all food. This method of feeding was used with two more rats, each of which was given only 0.2 gm. of the same sample of *p*-bromophenyl-*d*-cysteine. These animals likewise refused further food. The urine that could be collected from the animals was pooled. 179 mg. of acetyl derivative were isolated from the combined urines. The purified crystals possessed a rotation of $[\alpha]_D^{23} = +5°$ and a melting point of 149–152°.

$C_{11}H_{12}O_3NSBr$. Calculated, S 10.08, Br 25.12; found, S 9.94, Br 24.92

1 gm. of *p*-bromophenyl-*d*-cysteine having a rotation of $[\alpha]_D^{23} = -16°$ was mixed with 15 gm. of stock rat diet and the mixture was given *ad libitum* to a 270 gm. male rat. In 6 days the rat ate 10 gm. of the food, then refused all further food, and died on the 8th day. 125 mg. of acetyl derivative were isolated from the urine which had been collected. The isolated acetyl derivative had a melting point of 151° and a rotation of $[\alpha]_D^{26} = +4°$.

1 gm. of *p*-bromophenyl-*d*-cysteine having a rotation of $[\alpha]_D^{23} = -16°$ was powdered and suspended in 10 cc. of neutral olive oil. This suspension was injected intraperitoneally into a 270 gm. male rat at the rate of 2.5 cc per day for a period of 4 days. The urine was collected for a 6 day period. 270 mg. of acetyl derivative were isolated from the urine. The isolated derivative had a melt-

ing point of 152° and a rotation of $[\alpha]_D^{26} = +11°$. The nitrogen content of the compound was 4.37 per cent which agreed with the calculated value of 4.40 per cent.

SUMMARY

An unequivocal demonstration of the direct acetylation *in vivo* of *p*-bromophenyl-*d*-cysteine has been presented. The excretion of the *d*-mercapturic acid was observed after the feeding of the *p*-bromophenyl-*d*-cysteine. Partial inversion was indicated by the excretion of some *l*-mercapturic acid, the amount depending on the rate of administration of the *p*-bromophenyl-*d*-cysteine.

BIBLIOGRAPHY

1. du Vigneaud, V., and Irish, O. J., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **109**, p. xciv (1935); *J. Biol. Chem.*, **122**, 349 (1937–38).
2. du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131**, 273 (1939).
3. du Vigneaud, V., Wood, J. L., and Irish, O. J., *J. Biol. Chem.*, **129**, 171 (1939).
4. Friedmann, E., *Beitr. chem. Physiol. u. Path.*, **4**, 486 (1903).
5. Wood, J. L., and du Vigneaud, V., *J. Biol. Chem.*, **130**, 109 (1939).

STUDIES ON PLASMA PHOSPHATASE ACTIVITY DURING EMBRYONIC AND TUMOR GROWTH

By LEOPOLD WEIL

(From The Biochemical Research Foundation of the Franklin Institute, Philadelphia)

The analogy between embryonic and tumor growth frequently emphasized by investigators was found by us to exist in connection with the plasma proteinase activity of albino rats (1). We extended, therefore, our investigations in this direction to the activity of plasma phosphatase; this enzyme, as we have shown recently (2), plays an important part in the fat metabolism of the albino rat. No such relationship between embryonic and tumor growth was found with respect to the plasma phosphatase activity, since practically no enzymatic changes were observed during tumor growth, while marked enzymatic changes occurred during embryonic and normal growth.

EXPERIMENTAL

The ultramicromethod developed by us for the estimation of the plasma phosphatase activity (2) was employed. Only the full activities of the plasma phosphatase, activated by Mg'', are given, since the initial activities, without Mg'', run parallel with the former ones and do not alter the interpretation of our results.

Influence of Pregnancy and Embryonic Growth on Plasma Phosphatase Activity

The possible influence of pregnancy on the plasma phosphatase activity of sheep and cows has been studied by Wilson and Hart (3) and Auchinachie and Emslie (4). The results of these authors, however, did not show convincing regularities. An increased serum phosphatase in pregnant human patients was found by Cayla and Fabre (5) and by Bodansky, Campbell, and Ball (6).

The reinvestigation of this problem on rats, therefore, appeared desirable.

Mature albino rats were used and the plasma phosphatase activity was observed before pregnancy, during pregnancy, and also during the postpartum period. Fig. 1, which presents the results obtained, shows clearly that the plasma phosphatase activity of the rats remained practically constant as pregnancy progressed; however, about 6 days before parturition the enzyme activity dropped markedly and remained so until about 4 days after parturition, at which time a sharp increase in the plasma phosphatase activity could be observed. The latter rise continued and reached a maximum about 3 weeks after parturition.
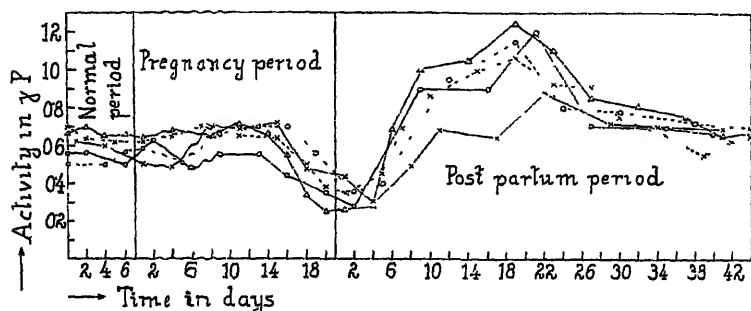
Fig. 1. Activity of plasma phosphatase

This maximum, as may be seen in Fig. 1, is much greater than the values in the normal and the pregnant rat. Thereafter the enzyme level decreased markedly and returned to the original plasma phosphatase values.

While we do not attempt to offer any explanation for the sharp drop in the plasma phosphatase activity shortly before parturition, we should like to point out that the marked increase in the plasma enzyme activity after parturition may be connected with the lactation period, which involves an increased milk fat production. In the light of our previous findings (2) the mobilization of the mechanism for this may well be connected with an increased plasma phosphatase activity. This assumption seems to be supported by the observation of Folley and White (7) who found that when the milk secretion was increased by administering thyroxine

the serum phosphatase activity was also slightly increased. The successive drops in the enzyme level to the normal values are possibly due to the decrease in lactation, transitory to the normal state of the rat.

Investigations were also undertaken to follow the plasma phosphatase activity of rats during normal growth, starting from the embryonic state. From rat embryos at various stages of development blood samples could be taken only once, since the embryos had to be sacrificed. Simultaneously samples of pla-

TABLE I

Plasma Phosphatase Activity, in Micrograms of P, of Rat Embryos and Placenta Blood

Blood plasma	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V
Embryo ..	0.16	0.20	0.24	0.28	0 18
Placenta.. .	0.18	0.18	0.24	0.26	0.20

TABLE II

Plasma Phosphatase Activity, in Micrograms of P, of Young Rats of Same Litter at Various Ages

Litter No.	Age in days								
	2	5	9	12	19	27	30	37	44
I	0.20	0.34	0.28	0.24	0.20	0 24	0.38	1.20	1.16
II	0.22	0.20	0.24	0.20	0.22	0.26	0.40	1.10	1.20
III	0.18	0.20	0.20	0.22	0 20	0.32	0.52	1 04	1.02

centa blood were taken and compared with that of the embryos. As Table I indicates, the plasma phosphatase values were practically the same, but, compared with the enzyme values obtained with adult rats, they were very low.

In another experiment, the plasma phosphatase activities of young rats were measured after birth. Here too blood samples could be taken only once. Results from the same litter only were compared. To avoid any possible dietary influence (2) at the time the young rats discontinued suckling and started to consume the food, Purina Dog Chow, given to the mother rat, powdered milk was substituted for the Purina Dog Chow. The results

presented in Table II show that young rats belonging to the same litter possess a very low plasma phosphatase activity, as do rat embryos (see Table I). However, at the age of about 1 month a remarkably sharp rise occurred in the plasma enzyme activity.

Since the sudden rise in the enzyme level of rats about 1 month old happened to coincide with the time at which the young rats started to consume a larger quantity of powdered milk, it was necessary to prove that this increase was not due to the dietary change from the milk of the rat to powdered milk (cow's). To decide this question, rats of the same litter 2 weeks old (at which age they still possess a low plasma phosphatase activity) were fed artificially with a solution of powdered milk every 4 hours over a period of 3 days. The results obtained, compared with those of the control rats fed on rat's milk, are as follows:

Powdered milk				Controls			
Rat No.. ... I	II	III	IV	V	VI	VII	VIII
0.20	0.30	0.24	0.24	0.24	0.22	0.26	0.26

The consumption of powdered milk solution by rats 2 weeks old did not produce an increased plasma phosphatase activity, as the above figures show, and the sharp rise in the enzyme level, as can be seen from Table II, is not connected with the slight differences in the composition of rat's and cow's milk, but is closely associated with the normal development of the rat. Similar observations were made by Stearns and Warweg (8) in humans and by Bodansky (9) on young dogs. Experiments which are under way indicate that the high plasma phosphatase activity of the rat 1 month old decreases gradually with the maturity of the rat, but remains above the original low enzyme value. Only extended experiment can reveal the characteristic behavior of plasma phosphatase, covering the whole life span of the rat, during the various phases of growth.

Influence of Tumor Growth on the Plasma Phosphatase Activity of Rats

Ten rats of about 200 gm. in weight were inoculated on both sides with Jensen sarcoma, and five rats were used as controls.

The food used was Purina Dog Chow. The plasma phosphatase activity was measured at frequent intervals (two or three times a week) over a period of 2 months. All of the inoculated rats showed rapid tumor growth, but after about 1 month, in four of the tumor-bearing animals the tumor growth had stopped and after the end of the 2nd month the tumor regressed almost completely. By this coincidence we had an opportunity to study the possible influence of tumor regression on the plasma phosphatase activity of the albino rats.

Our investigations have shown that no significant changes in the plasma phosphatase activity were observed during the entire experimental period in tumor-bearing rats or in regressing tumor rats or in the controls. Thus we have not been able to observe any lowering of the plasma phosphatase activity level in tumor-bearing rats as reported by Sure, Kik, Buchanan, Thatcher, and DeGroat (10) but find ourselves in agreement with the observations made on human patients by other workers (11–14). Because of the negative findings of these investigations it was deemed unnecessary to present them in table form.

The similarity observed between embryonic and tumor growth of rats in respect to plasma proteinase (1) thus does not exist in the case of the plasma phosphatase activity.

SUMMARY

The plasma phosphatase activity of albino rats remained unchanged from the beginning of pregnancy until about 6 days before parturition, when a marked drop in the enzyme level was observed. This low activity remained so for about 4 days after parturition, after which a sharp rise was observed, reaching a maximum in about 3 weeks after parturition. The maximum reached was much higher than the original normal enzyme level. The plasma phosphatase activity then decreased gradually to its original level. The blood plasma of rat embryos and the placenta blood plasma showed a very low phosphatase activity. Young rats belonging to the same litter possessed a low plasma phosphatase activity until they reached the age of about 1 month, at which time a sharp rise in the plasma enzyme activity was observed. This increase was shown not to be attributable to dietary changes. Rats with Jensen sarcoma did not show any change in their

plasma phosphatase activity during tumor growth nor did the regression of the sarcoma have any influence on the plasma enzyme activity.

BIBLIOGRAPHY

1. Weil, L., and Russell, M. A., *J. Biol. Chem.*, **126**, 245 (1938).
2. Weil, L., and Russell, M. A., *J. Biol. Chem.*, **136**, 9 (1940).
3. Wilson, L. T., and Hart, E. B., *J. Dairy Sc.*, **15**, 116 (1932).
4. Auchinachie, D. W., and Emslie, A. R. G., *Biochem. J.*, **27**, 351 (1933).
5. Cayla, J., and Fabre, F., *Compt. rend. Soc. biol.*, **120**, 748 (1935).
6. Bodansky, M., Campbell, K., and Ball, E., *Am. J. Clin. Path.*, **9**, 36 (1939).
7. Folley, S. J., and White, P., *Proc. Roy. Soc. London, Series B*, **120**, 346 (1936).
8. Stearns, G., and Warweg, E., *J. Biol. Chem.*, **102**, 749 (1933).
9. Bodansky, A., *J. Biol. Chem.*, **104**, 717 (1934).
10. Sure, B., Kik, M. C., Buchanan, K. S., Thatcher, H. S., and DeGroat, A. F., *Biochem. J.*, **29**, 1508 (1935).
11. Bodansky, A., and Jaffe, H. L., *Arch. Int. Med.*, **54**, 88 (1934).
12. Schoonover, J. W., and Ely, J. O., *Biochem. J.*, **29**, 1809 (1935).
13. Franseen, C. C., and McLean, K., *Am. J. Cancer*, **24**, 299 (1935).
14. Woodward, H. Q., and Higinbotham, N. L., *Am. J. Cancer*, **31**, 221 (1937).

A MICROMETHOD FOR THE DETERMINATION OF GLYCOCYAMINE IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

BY JACOB W. DUBNOFF AND HENRY BORSOOK

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

In the two following communications (1, 2) evidence is presented that glycocyamine is a normal precursor of creatine in the animal body. These studies required a satisfactory micromethod for the determination of glycocyamine. The most reliable method described in the literature consists in adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of arginine from the eluate by repeated adsorption on permutit, and colorimetric determination of the remaining glycocyamine by means of the Sakaguchi reaction. There are only two substances which are common in biological fluids and which give an intense color in the Sakaguchi reaction. These are arginine and glycocyamine. This method was first introduced by Weber (3) and was modified by Bodansky (4) and by Davenport and Fisher (5).

In our hands even the latest version of the method, that described by Davenport and Fisher, had the following shortcomings: it was laborious and time-consuming, the adsorption of the glycocyamine on the Lloyd's reagent was incomplete, further losses of glycocyamine occurred in the repeated treatment with permutit (Davenport and Fisher report losing only 10 per cent in three adsorptions; with the permutit available to us we lost 80 per cent), and the color developed was unstable. Furthermore, the amount of glycocyamine lost on the permutit varied according to the amount of arginine present, the less arginine the greater the loss of glycocyamine.

All these disadvantages have been removed in the method described below. It is the first method in which glycocyamine

381

added to blood or urine can be determined quantitatively, even in concentrations as low as 0.1 mg. per cent. 2 to 5 ml. are sufficient for an analysis. An indication of the speed and convenience of the method is that twenty to forty analyses can be carried through simultaneously in about 2 hours.

Reagents—

0.02 M sodium-potassium phosphate solution at pH 7.0 (Sørensen buffer); or 0.3 per cent sodium chloride solution.

Permutit.

A stock solution of 0.2 per cent α-naphthol in absolute alcohol, diluted with water to 0.04 per cent before use.

40 per cent solution of urea in water.

Hypobromite solution made as follows: 0.66 ml. of liquid bromine (specific gravity 3.0) in 100 ml. of 5 per cent sodium hydroxide.

Procedure

Urine is diluted 5 to 10 times, according to its concentration. Blood may be deproteinized either by adjusting the pH to 6.0, 5-fold dilution with water, boiling and filtration, or by precipitation of the proteins with sodium tungstate and sulfuric acid according to the procedure of Folin and Wu, with the modification that for each ml. of blood, 2 ml. of water and 1 ml. each of the 10 per cent sodium tungstate and 0.66 N sulfuric acid are used. Tissue extracts are diluted with water until the final solution contains 1 gm. of tissue (fresh weight) in 40 ml. of suspension. The pH is adjusted to 6.0, and the suspension immersed in a boiling water bath for 10 minutes, cooled, and filtered. The analysis is carried out on the deproteinized filtrate.

Any arginine which may be present is removed by sending the solution through a column of permutit contained in the stem of a funnel made of two pieces of glass tubing. The dimensions are as follows: upper piece 100 mm. long, 15 mm. external diameter; lower piece 100 mm. long, 7 mm. external diameter (5 mm. internal diameter). The bottom end of the funnel is constricted slightly.

The dimensions of the lower narrow tubing should be as close as possible to those given above. If the tubing is narrower, the filtration will be slow; if it is much wider the reduced height of the

column of permutit may allow some arginine to escape adsorption. The lower end of the funnel is stoppered loosely with a small piece of absorbent cotton just sufficient to hold back the permutit. 0.9 gm. of permutit is poured in and tapped gently to remove entrapped air. It should form a column about 85 mm. tall. A test-tube graduated at 10 ml. is placed under the end of the funnel.

5 ml. of the solution to be analyzed, obtained as described above, are pipetted into the funnel. After all of this solution has passed below the top of the permutit column, 5 ml. of either the 0.02 M phosphate buffer or of the 0.3 per cent sodium chloride solution are pipetted in above the permutit. Each 5 ml. portion of solution takes about 15 minutes to pass through the column. When the second 5 ml. portion has passed through, the filtrate in the graduated test-tube below is made up to the 10 ml. mark with water and then shaken.

Washing the permutit column with the phosphate or sodium chloride solution removes the small amount of glycocyamine which remains in the permutit column after the 5 ml. of the solution under analysis have passed through. The dilute phosphate buffer and the sodium chloride solution are equally effective. We prefer the sodium chloride solution, because it is simpler to make up and also because a faint turbidity is sometimes obtained in the final filtrate when the phosphate solution is used.

Different batches of permutit may differ in their affinity for arginine and glycocyamine. We have mentioned above that Davenport and Fisher lost only 10 per cent of the glycocyamine present in three successive treatments with fresh permutit, whereas we lost 80 per cent. It may be advisable therefore before a fresh batch of permutit is used in the procedure described to ascertain the height of the column and amount of permutit necessary to remove all the arginine without loss of glycocyamine. The prescription we have given allows some latitude and it may cover all the variations in permutit now sold in this country.

The permutit can be regenerated after it has been used by the following procedure. It is washed several times with tap water, thrown on a Buchner funnel, an equal volume of 25 per cent sodium chloride solution passed through, and then washed with distilled water until the wash water is chloride-free.

The development of the color by means of the Sakaguchi reaction

and the colorimetry may be carried out with any convenient aliquot so long as the proportions of the reagents are kept the same. The quantities of reagents given below are for 2 ml. aliquots which we have been using. Twenty to forty analyses can be carried through simultaneously. Test-tubes containing the aliquots of the solutions to be analyzed, inserted in a rack, are

TABLE I

Determination of Glycocyamine in Aqueous Solutions Containing Different Amounts of Interfering Substances

All values are concentrations measured in mg. per cent.

The concentrations of the added substances were those in the final mixtures before they were sent through the permutit columns. The final filtrates contained only half the concentrations of glycocyamine shown. Accordingly the standards were diluted 1:1 with 0.02 M phosphate buffer solution.

Arginine added	Creatine added	Glycocyamine added	Glycocyamine found by analysis
35	10	3 0	3.02
35	5	3.0	3.01
35	1	3.0	3.01
35	0	3.0	3.00
35	10	1.0	1.01
35	5	1.0	1.02
35	1	1.0	1.00
35	0	1.0	1.00
35	10	0.1	0.10
35	5	0.1	0.10
35	1	0.1	0.10
35	0	0.1	0.10
70	25	0.1	0.10
35	25	0.1	0.10
18.5	25	0.1	0.09
0	25	0.1	0.09

placed in an ice water bath. In this bath also are the α-naphthol (0.04 per cent), urea, and hypobromite solutions. After 5 to 10 minutes 0.4 ml. of the α-naphthol solution is added to each tube, the contents shaken, 0.4 ml. of the chilled urea solution added, and the contents shaken again. After another 5 minute interval 0.2 ml. of the chilled hypobromite solution is added, the contents immediately mixed by vigorous shaking, and the tube replaced in the ice bath.

The color develops slowly, attaining its maximum intensity in about 20 minutes, after which (so long as the solution is in the ice bath) it remains constant for at least 1 hour. Before the color is measured, the solution is warmed to room temperature by shaking in water for $\frac{1}{2}$ minute and the dissolved gas removed by vigorous tapping for 20 seconds. If the colored solution is left at room temperature for $\frac{1}{2}$ hour or more, the color slowly fades.

TABLE II

Determination of Glycocyamine in Ringer's Solution and in Rat Heart and Kidney Extracts to Which Different Quantities of Arginine and Glycocyamine Were Added

All values are concentrations measured in mg. per cent.

The concentrations of added glycocyamine and the experimental values given were those in the final filtrates, *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution. The concentrations of added arginine were those before the solutions had passed through the permutit.

Arginine added	Glyco-cyamine added	Glycocyamine					
		Ringer's solution		Kidney extract		Heart extract	
		Found	After subtracting blank value	Found	After subtracting blank value	Found	After subtracting blank value
0	0			0.04	0	0.08	0
35	0	0	0	0.04	0	0.08	0
35	1.50	1.53	1.53	1.59	1.55	1.56	1.48
0	0.75	0.765	0.765	0.79	0.75	0.85	0.77
35	0.75	0.80	0.80	0.82	0.78	0.84	0.76
35	0.375	0 41	0.41	0.44	0.40	0.45	0.37
35	0 075	0.08	0.08	0 11	0.07	0.15	0 07

With each group of analyses the color is developed simultaneously in four standard solutions containing 0, 0.25, 0.5, and 1.0 mg. per cent of glycocyamine.

To measure the color we have used a spectrophotometer or a colorimeter. With either instrument the best light is that at approximately 0.525 μ. There is a linear relationship between the concentration of glycocyamine up to 1 mg. per cent and the intensity of the color when it is measured with light near to 0.525 μ.

Tables I to IV indicate the sensitivity and reliability of the method described above. Table I gives some typical results

with prepared mixtures, in aqueous solution, containing varying amounts of glycocyamine and of two interfering substances, arginine and creatine.

Table II summarizes some tests of the method with Ringer's solution and two tissue extracts. These tests were necessary, even after the results in Table I were obtained, because the equilibrium relation between the glycocyamine adsorbed on permutit and that remaining in solution is affected by the concentration of

TABLE III

Determination of Added Glycocyamine and of Added Arginine in Human Urine (Diluted 5-Fold with Water)

All values are concentrations measured in mg. per cent

The concentrations of added glycocyamine and the experimental values found were those in the filtrates; *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution   The concentrations of added arginine were those before the solutions had passed through the permutit.

Glycocyamine added	Arginine added	Glycocyamine	
		Found	After subtracting amount originally present
0	0	0.21	0
0	22	0.21	0
1.0	0	1.26	1.05
0.5	0	0.72	0.51
0.5	30	0.71	0.50
0.5	22	0 70	0.49
0.5	10	0 69	0.48
0 25	0	0.46	0.25

salt. The reliability of most of the experimental results described in the next communication (1) depends on the accuracy of this analytical method. The data in Table II show that the agreement between theoretical and experimental values is satisfactory.

The determination of glycocyamine in urine is one of the most severe tests to which an analytical method for glycocyamine can be subjected. Urine contains relatively high concentrations of interfering substances such as urea, ammonia, creatinine, and amino acids including arginine. The rigor of the test was in-

creased by adding to the urine varying amounts of arginine and glycocyamine. Table III shows that the same satisfactory agreement is obtained between theoretical and experimental values as in simple aqueous solutions. This is also the case with blood (Table IV).

TABLE IV

Determination of Glycocyamine Added to Human Blood

All values are given in mg. per cent.

Varying amounts of a 10 mg. per cent glycocyamine solution and of a 20 mg. per cent arginine solution were added to 4 ml. aliquots of whole human blood. To these mixtures water was added so that the final dilution of the blood was the same in each case; *i.e.*, 4 ml. of blood and 8 ml. of water or of the combined glycocyamine and arginine solutions. 4 ml. of 10 per cent sodium tungstate followed by 4 ml. of 0.66 N H_2SO_4 were then added to each mixture. The analyses were carried out on the filtrates. The concentrations of glycocyamine and arginine in these filtrates were further reduced by one-half in the analytical procedure.

Concentration ÷ 2 in Folin-Wu filtrate		Concentration of glycocyamine in Folin-Wu filtrate	
Glycocyamine added	Arginine added	Found	After subtracting amount originally present
1.0	0	0.99	0.95
0.5	0	0.55	0.50
0.2	0	0.24	0.19
0.1	0	0.15	0.10
0	0	0.05	0
0.5	2.0	0 55	0.50
0.5	1.5	0.55	0.50
0.5	1.0	0.54	0.49
0.5	0.5	0.54	0.49
0	2.0	0.05	0

SUMMARY

A micromethod is described for the determination of glycocyamine in biological fluids and tissue extracts. The advantages of this method over those previously described are that added glycocyamine is recovered quantitatively; it is faster and more convenient.

BIBLIOGRAPHY

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138**, 389 (1941).
2. Borsook, H., Dubnoff, J. W., Lilly, J. C., and Marriott, W., *J. Biol. Chem.*, **138**, 405 (1941).
3. Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930); **88**, 353 (1930).
4. Bodansky, M., *J. Biol. Chem.*, **115**, 641 (1936).
5. Davenport, H. W., and Fisher, R. B., *Biochem J.*, **32**, 602 (1938).

THE FORMATION OF GLYCOCYAMINE IN ANIMAL TISSUES*

By HENRY BORSOOK and JACOB W. DUBNOFF

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

It was shown in preceding communications that glycocyamine is converted into creatine by surviving liver slices (1). Our findings indicated that the methylating agent is methionine or a derivative of methionine. Liver slices can methylate glycocyamine rapidly enough to permit assignment to the liver alone, if necessary, of the task of making good the loss of creatine and creatinine in the urine. This holds for the livers of all mammals studied. We found no evidence of this methylating mechanism in any other tissues, except possibly slight activity in the kidney. In the pigeon the kidney is as effective in this respect as the liver.

These experimental facts were corroborated (as far as the rat is concerned) by experiments on living animals with tracers. Bloch and Schoenheimer, using N^{15}, found that glycocyamine is readily converted to creatine (2). Du Vigneaud and his collaborators fed rats methionine with deuterium in the methyl group; after only 3 days a relatively large quantity of deuterium was found in the muscle creatine (3).

Glycocyamine has had a favored position among the possible precursors of creatine. It is nearer to creatine structurally than any other precursor which has been proposed; and its convertibility to creatine in the living organism has been proved. The case against glycocyamine rested on two arguments: (1) that only a small fraction, 5 to 15 per cent, of administered glycocyamine is methylated, and (2) that glycocyamine had not been found as a normal constituent of animal tissues. It has, however, been isolated by Weber from human and dog urine (4).

---

* A summary of this work has appeared (*Science*, 91, 551 (1940)).

The findings with surviving liver slices and the tracer studies reinstated glycocyamine, more firmly than before, as a possible normal precursor of creatine. The quantity of creatine synthesized daily is so large that, if glycocyamine is its normal precursor, an active mechanism for the formation of glycocyamine must exist. We undertook a search for this mechanism.

The first necessity was an adequate analytical method. Such a method is described in the preceding communication (5).

With it we have found that kidney slices rapidly form glycocyamine from arginine and glycine. All other tissues tested are negative in this respect.

This interaction of arginine and glycine is also catalyzed by thoroughly macerated cell-free kidney tissue suspended in a phosphate buffer solution.

The formation of glycocyamine from arginine and glycine is a new biochemical reaction which may be called "transamidination." We propose that the enzyme be designated "glycine-transamidinase." The discovery of this reaction provides direct proof that arginine and glycine are precursors of creatine.

Bloch and Schoenheimer fed ammonia containing N^{15} to rats and later found the isotope in the amidine nitrogen of creatine. After glycine containing N^{15} was fed, the isotope was found in creatine in the sarcosine nitrogen. In a later communication the same authors presented more direct evidence (again obtained by the use of N^{15}) which confirmed the findings we had reported that glycocyamine is formed by the transfer of the amidine group from arginine to glycine. They fed $l(+)$-arginine with N^{15} in the amidine group to rats (6); afterwards the creatine in the muscles had a far higher isotopic content than after the administration of isotopic ammonia, urea, or any other amino acid except glycine. It was so high that they considered that the amidine group of creatine must have originated from arginine.

The present communication contains the details of our experimental procedure, our findings on the effects of a fairly large number of amino acids and derivatives of arginine and of glycine, surveys of the capacity for glycine transamidination of the organs of a number of common experimental animals, and the results of some studies of the effect of concentration of reactants, pH, and time on the rate of transamidination.

Technique and Results

The tissue slice technique used and the details of the reaction vessels are described in a previous communication (7).

At the end of an experiment the contents of the reaction vessels were transferred with the slices to test-tubes graduated at 20 ml.; the vessels were washed with three 2 ml. portions of 0.02 M phosphate buffer solution at pH 6.0 and the washings added to the main solution. The pH was adjusted finally to 6 with a drop of 0.5 N hydrochloric acid. The test-tubes containing the slices, the main solutions, and washings were kept in a boiling water bath for 10 minutes, after which they were cooled to room temperature, and made up to the 20 ml. mark with water and mixed by shaking. These solutions were then filtered. 5 ml. of the clear protein-free filtrate were analyzed for glycocyamine by the procedure described in the preceding communication (5).

The coagulated slices and protein in each test-tube were transferred to small glass weighing dishes, heated overnight at 105°, and, after cooling in a desiccator, were weighed.

Table I is the detailed protocol of a typical experiment. A significant amount of glycocyamine is formed when arginine alone is added to the Ringer's solution. Glycine without arginine also leads to a slight increase in glycocyamine. When both amino acids are added together, the increase in glycocyamine is more than 10 times the increase with arginine alone. The increases obtained with arginine alone and with glycine alone indicate either the presence of these amino acids in the free state (more glycine than arginine) or their formation in small amounts by autolysis within the slices.

In the experiment whose results are recorded in Table I, and in a number of others, creatine analyses were carried out with the specific bacterial (NC) enzyme of Dubos and Miller (8, 9). No evidence of creatine formation was found.

The steps in the proof that the substance we were measuring was glycocyamine were as follows: Autoclaving in acid solution produced a substance which was adsorbed by Lloyd's reagent and gave a positive test with the Jaffe reagent. This chromogenic material was not digested (before autoclaving) by the NC bacteria of Dubos and Miller under conditions in which creatine and creatinine were completely digested. A strongly positive Saka-

guchi test was obtained in the unautoclaved solution after all the arginine was removed by exhaustive adsorption on permutit. The depth of color which the unknown solution gave with the Jaffe reagent (after autoclaving) corresponded, assuming it to be glycocyamine, to the intensity of color it gave in the Sakaguchi reaction after removal of the arginine. A liter of solution was

TABLE I

Protocol of Typical Experiment Showing Formation of Glycocyamine from Arginine and Glycine by Rat Kidney Slices

Ringer's solution, 38°, 4 hours.

Dry weight of slices (1)	Ringer's solution (2)	0.02 M arginine in Ringer's solution (3)	0 04 M glycine in Ringer's solution (4)	Concentration of glyco-cyamine in aliquot taken for analysis (5)	Glycocyamine found per 100 gm. fresh tissue (6)
mg.	*ml.*	*ml.*	*ml.*	*mg. per cent*	*mg.*
26.4	4			0.02	6
30.4	4			0.04	11
22.5	3	1		0.10	36
21.0	3	1		0.11	42
20.0	3		1	0.04	16
22.0	3		1	0.05	18
23.6	2	1	1	1.03	349
21.4	2	1	1	0.97	363

The figures in Column 6 are obtained by multiplying those in Column 5 by 8000 and dividing them by the dry weight of the tissue in mg. (Column 1). The figure 8000 is obtained as follows: the solution is diluted 5-fold before analysis; in the course of analysis it undergoes a further 1:1 dilution, the results in Column 5 expressed as mg. per cent must be divided by 25, since there were only 4 ml of the original reaction solution; to express the results on the basis of 100 gm. of fresh tissue, the factor $100,000/5 \times W$ is used, W being the dry weight in mg. given in Column 1. The factor therefore is $5 \times 2 \times 1/25 \times 100,000/(5 \times W) = 8000/W$.

now collected in which kidney slices had acted upon arginine and glycine, and which contained, according to analysis, about 50 mg. of glycocyamine. The glycocyamine was isolated by adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of the arginine by repeated adsorption with permutit, and crystallization from glacial acetic acid as glycocyamine acetate. These crystals were the characteristic needles and thin prisms (10).

The free glycocyamine was regenerated from the acetate by boiling in dilute aqueous solution and crystallized by evaporation of the water.   25 mg. of crude glycocyamine were thus obtained. It was thrice recrystallized from water, with a final yield of 11 mg. of the pure dry material which was analyzed.   It gave the following figures.[1]

Observed.	C 30.8, H 5.95, N 35 8
Theoretical for glycocyamine.	" 30 8, " 6.0, " 35.9

TABLE II

Formation of Glycocyamine by Rat Kidney Slices from Arginine and Glycine or Glycine Derivatives

Glucose-Ringer's solution, 3 hours, 38°.   Concentration of arginine 0.005 M; glycine or derivatives 0.01 M.

Arginine	Glycine or glycine derivative	Glycocyamine found per 100 gm. fresh tissue
		mg.
–		8
+		35
–	Glycine	19
+	Betaine	44
+	Glutathione	296
+	Glycine	382
+	" anhydride	8
+	Glycylglycine	436
+	Glycolic acid + ammonia	6
+	Hippuric acid	35
+	Leucylglycine	254
+	Sarcosine	109
+	" anhydride	6

Table II summarizes the relative effectiveness of glycine and some glycine derivatives as precursors of glycocyamine.   The effect of the glycine peptides is accounted for on the hypothesis that these are first hydrolyzed and that it is the free glycine which reacts with arginine to form glycocyamine.   The argument is as follows: The rate of glycocyamine formation is proportional to the concentration of free glycine (Table VIII).   When glycine

[1] We are indebted to and wish to thank Dr. A. J Haagen-Smit for these analyses.

peptides were the source of the glycine, the concentration of free glycine was initially 0 and only in the course of the 3 hour experimental period did it approximate 0.01 M, whereas when glycine itself was added the initial concentration was 0.01 M. Hence smaller amounts of glycocyamine were formed from glutathione, leucylglycine, and hippuric acid than from the same initial concentration of glycine. Glycylglycine gave a higher value than glycine, because on hydrolysis it yields 2 molecules of glycine and as a result the concentration of glycine rose well above 0.01 M before the end of the 3 hour period.

It follows, if the above is the correct explanation of the effect of glycine peptides, that rat kidney contains a dipeptidase for leucylglycine, an enzyme which liberates glycine from glutathione, and no enzymes capable of forming free glycine at a significant rate from betaine, glycine anhydride, or hippuric acid.

The results with sarcosine indicate that this substance is demethylated without deamination in rat kidney. Separate analyses showed that no creatine was formed; this proved that demethylation of the sarcosine had occurred prior to the transamidination. The kidney contains, therefore, an enzyme which demethylates sarcosine.

These findings with sarcosine are complemented by those of Bloch and Schoenheimer (2, 11) who, using N^{15} as a tracer, found that sarcosine is converted to glycine *in vivo* and that in the course of the demethylation the glycine nitrogen originally attached to the carbon chain is not replaced. Their experiments therefore excluded intermediate deamination of sarcosine in the course of its conversion to glycine.

Analogous to its inability to hydrolyze glycine anhydride the kidney is unable to hydrolyze sarcosine anhydride.

The negative result shown in Table II with glycolic acid and ammonia indicates that rat kidney is unable to form glycine from these two substances at a significant rate.

The experiments summarized in Table III revealed that rat kidney evidently is able to synthesize arginine from citrulline. The other possible amidine donators which were tested, guanidine, ornithine, and urea, were negative.

The positive result with citrulline was retested in a number of more adequately controlled experiments. A group of typical

TABLE III

Formation of Glycocyamine by Rat Kidney Slices from Glycine and Arginine or Other Possible Donators of Amidine Group

Glucose-Ringer's solution, 3 hours, 38°. Concentration of glycine 0.01 M; of arginine or other amidine donators 0.005 M.

Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
		mg.
−		35
−	l(+)-Arginine	68
+	"	498
+	l(+)-Citrulline + ammonia	330
+	Guanidine	16
+	d(−)-Ornithine + ammonia	12
+	Urea	33

TABLE IV

Formation of Glycocyamine from Glycine and Citrulline by Rat Kidney Slices and by Cell-Free Macerate of Rat Kidney

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0, 3 hours, 38°. Glycine 0.01 M; amidine donator (ammonia, arginine, citrulline, or ornithine) 0.005 M.

	Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
			mg.
Kidney slices	−		22
	+		31
	−	Ammonia	18
	−	Arginine	68
	−	Citrulline	58
	+	Arginine	357
	+	Citrulline	200
	+	" + ammonia	227
	+	Ornithine + "	8
Cell-free macerate	−		30
	+	Arginine	120
	+	Citrulline + ammonia	59

results is shown in Table IV. Citrulline was only slightly less effective than citrulline plus ammonia.

Included in Table IV are some typical results obtained with a

cell-free macerate of kidney. This suspension was less active than an equivalent amount of kidney tissue in the form of slices but qualitatively the results were in every respect the same.

The suspension was made by the homogenizing procedure of Potter and Elvehjem (12). The kidney was stripped of its capsule, split down the longitudinal axis, the pelvic fat and the medulla cut away, the remainder homogenized with 4 times its weight of 0.01 M phosphate buffer at pH 7.0, and the resulting suspension passed through gauze. Such a suspension retains all its transamidinase activity for at least 2 months if kept in an ice box.

The optimum pH of glycine-transamidinase is in the neighborhood of pH 7.0 (Table V).

TABLE V

Effect of pH on Activity of Glycine-Transamidinase

38°, 4 hours.   Glycine 0.01 M; arginine 0.005 M.

pH	Glycocyamine formed per 100 gm. fresh tissue
	mg.
6.0	214
6.5	259
7.0	320
7.5	278
8.0	246

The activity of glycine-transamidinase in cell-free solution is unaffected by 0.001 M potassium cyanide or by carrying out the reaction *in vacuo*. For example, one extract under the same conditions as those described above formed 67 mg. of glycocyamine per 100 gm. of fresh tissue; in the presence of 0.001 M potassium cyanide it formed 80 mg. and anaerobically, 72 mg.

The following amino acids and amides were tested with rat kidney slices and arginine as possible precursors of the glycine radical in glycocyamine. The initial concentration in every case was 0.005 M. They were all negative: d-alanine, dl-alanine, l-asparagine, l-aspartic acid, l-cysteine, l-cystine, d-glutamic acid, d-glutamine, l-hydroxyproline, l-histidine, dl-isoleucine, l-leucine, d-lysine, dl-methionine, dl-norleucine, l-proline, dl-phenylalanine, dl-serine, d-threonine, l-tryptophane, l-tyrosine,

and *d*-valine. These negative results indicate that under the conditions of these experiments none of these amino acids is a precursor of glycine.

Glycine-transamidinase activity was found in the kidney of every animal tested except the frog (Table VI). Whenever activity was found in kidney slices, it was also found in the cell-free extract. The beef and sheep kidneys were used at least 24 hours after the animals were slaughtered; they were obtained in a butcher shop. In the cases of all the other animals the kid-

TABLE VI

Formation of Glycocyamine from Glycine and Arginine by Kidney Slices and by Cell-Free Macerate of Kidney of Various Animals

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0. Glycine 0.01 M; arginine 0.005 M. 38°, 3 hours.

Animal	Glycocyamine formed per 100 gm. fresh tissue by	
	Kidney slices	Cell-free macerate
	mg.	*mg.*
Beef. .		190
Cat .	93	32
Dog	281	480
Frog.....		0
Guinea pig...	38	14
Pigeon	27	16
Rabbit . .	187	160
Rat . ..	357	120
Sheep .. .		160

neys were removed immediately after the animals were killed and the extracts made soon afterwards. The negative results with extracts of frog kidney call for further study.

Liver slices and cell-free extracts of heart and of muscle of all the animals listed in Table VI were tested for glycine-transamidinase activity. Except in the case of the pigeon (see below) they were all negative. The blood, brain, intestine, and spleen of the rat were also examined; they were negative. Rat liver slices also gave negative results with glycine plus arginine, urea, or guanidine.

Before the advent of Weber's method no glycocyamine could be demonstrated in animal tissues. With this method glyco-

cyamine was detected in urine (4), in intestine, testes, and kidney (13).

Using our more sensitive method, we found glycocyamine to be widely distributed in the tissues of the rat. The concentrations (mg. per 100 gm. of fresh tissue) were blood 0.5 to 1; brain, heart, liver, skeletal muscle, and spleen 3 to 6; small intestine 10; kidney (cortex) 15 to 30.

The question arose whether transamidination in the kidney can be sufficiently rapid under physiological conditions to account for the total production of creatine in the body as indicated by the

TABLE VII

Rate of Glycocyamine Formation by Rat Kidney Slices at 38° from Arginine Initially 0.005 M and Glycine 0.01 M

Time	Glycocyamine per 100 gm. fresh tissue		
	Found	Formed	Average rate of formation per hr.
hrs.	*mg.*	*mg.*	*mg.*
0	32		
0.5	95	63	126
1.0	164	132	132
2.0	315	283	142
4.0	505	473	118
6.0	741	709	118

daily excretion in the urine of creatine and creatinine. A number of experiments were carried out to obtain what information we could on this point.

Table VII shows that the glycine-transamidinase in rat kidney slices remained practically unimpaired for 6 hours. This is in accord with the stability of the enzyme in cell-free extracts.

In Table VIII are some figures on the effect of the concentration of the reactants, arginine and glycine, on the rate of transamidination. With equal arginine and glycine concentrations from 0.001 to 0.0001 M the rate was approximately linear with respect to concentration.

We can estimate what the rate of glycocyamine formation in the kidney must be to make good the loss of tissue creatine which appears in the urine as creatinine. Two human kidneys

weigh approximately 300 gm. An average figure for the creatinine excreted in the urine in 24 hours is 1.7 gm. To make good this loss the average hourly production of glycocyamine which is necessary must be approximately 25 mg. per 100 gm. of tissue per hour.

This rate of glycocyamine formation would have been obtained in the experiments of Table VIII with concentrations of glycine and arginine between 0.0005 and 0.001 M. This is probably the concentration range of these amino acids in kidney. The basis of this estimate is as follows: The arginine concentration in dog

TABLE VIII

Variation in Rate of Glycocyamine Formation by Rat Kidney Slices with Different Concentrations of Arginine and Glycine

Ringer's solution, 4 hours, 38°.

Initial concentration		Glyco-cyamine formed per 100 gm. fresh tissue	Initial concentration		Glyco-cyamine formed per 100 gm. fresh tissue	Initial concentration		Glyco-cyamine formed per 100 gm. fresh tissue
Arginine	Glycine		Arginine	Glycine		Arginine	Glycine	
mole per l.	mole per l.	mg.	mole per l.	mole per l.	mg.	mole per l.	mole per l.	mg.
0.02	0.01	190	0.005	0.02	617	0.005	0.005	360
0.01	0.01	590	0.005	0.01	533	0.0025	0.0025	310
0.005	0.01	533	0.005	0.005	361	0.001	0.001	134
0.0025	0.01	535	0.005	0.0025	252	0.0005	0.0005	78
0.00125	0.01	303	0.005	0.00125	201	0.0001	0.0001	20
0.000625	0.01	201	0.005	0.000625	103			

blood is between 2.6 and 3.9 mg. per cent (14); we have found 2.0 to 5.0 mg. per cent in human blood. This range corresponds to 0.0001 to 0.00025 M. The concentrations of these amino acids in the kidney are certainly much higher than in blood, since the total free amino nitrogen in kidney and other tissues is 10 times or more that in blood (15, 16). The rates of glycocyamine formation recorded in Table VIII are sufficient therefore to make good the total loss of creatine from the tissues. This estimate is, of course, based on the assumption that the rate of glycocyamine formation in human kidney *in vivo* is of the same order as in rat kidney slices *in vitro*, which seems not unreasonable.

The experimental results given above, confirmed and complemented as they now are by the findings in studies with tracers, make a strong case for the following mechanism of creatine formation in animals: (a) arginine and glycine in the kidney form glycocyamine; (b) in the liver glycocyamine and methionine form creatine. The mechanisms for these reactions are widely distributed in mammals. The quantitative aspects of the data show that the transamidination mechanism for the formation of glycocyamine is fast enough to replace all the creatine lost from the body. Other mechanisms of creatine formation are not excluded; but the tracer studies indicate that the arginine-glycine-methionine mechanism is quantitatively the most important one *in vivo*.

The argument *contra* glycocyamine, that it had never been found as a constituent of animal tissues, antedated the development of suitable analytical methods. This argument is now completely disposed of by the detection of glycocyamine in practically all tissues.

The controversy whether arginine is a precursor of creatine (17, 18) may be considered as settled in favor of the affirmative. The path of its conversion to creatine, however, is different from what was generally believed. It was proposed that arginine was converted to glycocyamine by way of deamination and β oxidation; thus all of the nitrogen was derived directly from arginine. This made it necessary to explain away the evidence which suggested that glycine played some part in creatine formation, unless it be in the methylation of glycocyamine.

Bergmann and Zervas were the exception. They observed that "triacetyl anhydro arginine" and glycine ethyl ester reacted in the absence of water to give a fairly good yield of diacetyl glycocyamine ethyl ester (19); and the same arginine derivative with sarcosine ethyl ester gave diacetyl creatine ethyl ester (20). Their view was that, "der wesentliche Punkt der biologischen Kreatinbildung in einer direkten Umsetzung eines reaktionslustigen Argininabkömmlings mit einem Aminoäthanderivat beruht." This clear statement that the guanidine group of creatine arises *in vivo* by transamidination needs now to be modified in two respects: that an enzyme, glycine-transamidinase, and not a

reactive derivative or split-product renders the $-\text{C}\diagdown\!\!\!\!\genfrac{}{}{0pt}{}{\diagup\text{NH}_2}{\text{NH}}$ group
in arginine labile as it is in "triacetyl anhydro arginine." The enzyme arginase exerts a similar influence. The other modification is that glycine itself and not a derivative is the acceptor of the amidine group in the biological transamidination reaction.

We may now infer that one of the reasons for the essential character of arginine and methionine in the rat is that they participate in the formation of creatine. These amino acids, of course, also serve other functions, for example the rôle of arginine in urea formation and of methionine in other methylation reactions (3), in addition to their participation in the constitution of tissue protein.

The recent findings on essential amino acids for the chick indicate that arginine and glycine are required for creatine formation in this animal. Arnold *et al.* (21) reported that arginine is essential for rapid growth. This was confirmed by Klose *et al.* (22) who showed further that arginine is necessary for maintenance as well as growth. Recently Klose and Almquist reported that citrulline is as effective as arginine, whereas ornithine alone or with urea is ineffective (23). We have found (Tables III and IV) that glycocyamine is formed from citrulline and glycine, the citrulline presumably being first converted to arginine, while ornithine, with or without added ammonia, is completely negative.

The parallel between the amino acid requirements for creatine formation and for growth was extended further when Almquist *et al.* found that glycine is essential for the growth of the chick (24) and that creatine as a substitute for glycine is even more effective than glycine itself. Glycolic acid and betaine could not replace creatine (25).

We have examined the organs of the pigeon for transamidinase activity. Activity was found not only in the kidney but also in heart, liver, and skeletal muscle. The limiting amino acid appeared to be glycine; *i.e.*, nearly as much glycocyamine was formed when glycine alone was added as from glycine and arginine together. The differences between experimental and control were, however, small in absolute terms compared with those found in the kidneys of other animals. Although these

differences were greater than could be ascribed to analytical or sampling variations, we cannot consider our findings in the pigeon as established until more determinations have been made. For this reason the detailed figures are not presented here. We hope that other workers with facilities for a study such as this on birds may undertake the investigation. Our laboratory does not at present possess such facilities.

SUMMARY

1. Beef, cat, dog, guinea pig, pigeon, rabbit, rat, and sheep kidney form glycocyamine from arginine and glycine. This reaction is catalyzed by cell-free extracts of kidney as well as by surviving kidney slices.

2. It is proposed that this reaction be designated "transamidination," and the enzyme "glycine-transamidinase." The optimum pH of this enzyme is about 7.0. It is not affected by potassium cyanide nor by anaerobiosis.

3. Transamidination does not occur in the liver, heart, or skeletal muscle of the animals mentioned above; the blood, brain, and spleen of the rat were tested also and found inactive. It is possible that in the pigeon a low glycine-transamidinase activity resides in liver, heart, and skeletal muscle as well as in kidney.

4. Glycocyamine is also formed in the kidney from glycine and citrulline. Glycine plus ornithine (with or without ammonia), urea, or guanidine is negative in this respect.

5. A large number of amino acids, several amides, and anhydrides were tested as possible precursors of the glycine radical of glycocyamine. They were all negative, as was also glycolic acid plus ammonia.

6. Glycocyamine is formed from arginine and sarcosine. Evidence is presented that the sarcosine is first demethylated, thus being converted to glycine, indicating the presence of a demethylating enzyme in kidney. Sarcosine anhydride is negative.

7. The above findings, complemented by the tracer studies in the laboratories of Schoenheimer and of du Vigneaud, and in conjunction with our previous findings, prove the existence of the following mechanism of creatine formation in animals: arginine and glycine form glycocyamine in the kidney; the glycocyamine is methylated in the liver by methionine (or a derivative of methionine) to form creatine.

8. Quantitative aspects of the data indicate that all of the creatine formed in animals may normally be formed by this mechanism.

9. Evidence of the generality of transamidination is seen in the close parallel between the above findings and those on amino acids essential for the growth of the chick.

BIBLIOGRAPHY

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940); **134**, 635 (1940).
2. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 633 (1940).
3. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).
4. Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, **32**, 172 (1934); *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **109**, p. xcvi (1935)
5. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941).
6. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 785 (1940).
7. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 307 (1940).
8. Miller, B. F., and Dubos, R., *Proc. Soc. Exp. Biol. and Med.*, **35**, 335 (1936).
9. Dubos, R., and Miller, B. F., *J. Biol Chem.*, **121**, 429 (1937).
10. Jaffe, M., *Z. physiol. Chem.*, **48**, 430 (1906).
11. Bloch, K., and Schoenheimer, R , *J. Biol Chem.*, **135**, 99 (1940).
12. Potter, V. R., and Elvehjem, C. A., *J. Biol Chem.*, **114**, 495 (1936)
13. Bodansky, M., *J. Biol. Chem.*, **115**, 641 (1936).
14. Weber, C. J., *J. Biol. Chem.*, **88**, 353 (1930).
15. Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, **52**, 417 (1922).
16. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, **16**, 197 (1913–14).
17. Hunter, A., Creatine and creatinine, Monographs on biochemistry, London and New York (1928).
18. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, American Chemical Society monograph series, New York (1929).
19. Bergmann, M., and Zervas, L , *Z. physiol. Chem.*, **172**, 277 (1927).
20. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **173**, 80 (1928).
21. Arnold, A., Kline, O. L., Elvehjem, C. A., and Hart, E. B , *J. Biol. Chem.*, **116**, 699 (1936).
22. Klose, A. A., Stokstad, E L R., and Almquist, H. J., *J. Biol. Chem* , **123**, 691 (1938).
23. Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, **135**, 153 (1940).
24. Almquist, H. J , Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, **134**, 213 (1940).   Almquist, H. J., Mecchi, E., Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **134**, 465 (1940).
25. Almquist, H J., and Mecchi, E., *J. Biol. Chem.*, **135**, 355 (1940)

THE FORMATION OF GLYCOCYAMINE IN MAN AND ITS URINARY EXCRETION

By HENRY BORSOOK, JACOB W. DUBNOFF, JOHN C. LILLY, AND WILLIAM MARRIOTT

(*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*)

Glycocyamine was first isolated from human and dog urine and identified by Weber (1–3). He supported the view that glycocyamine is a normal precursor of creatine and that its appearance in urine (2) is "an overflow phenomenon of an intermediate metabolic product . . ." He expressed no views on the mechanism of its formation.

Glycocyamine is formed by the interaction of arginine and glycine in the kidney (and probably only in that organ) of the cat, dog, guinea pig, rabbit, and rat (4). In order to ascertain whether this reaction occurs in man we have studied the effect of arginine and glycine, ingested separately and together, on the glycocyamine content of urine and blood.

The subjects were seven normal men. The methods used for the determination of glycocyamine in urine and in blood have been described (5).

The first experiments consisted in comparing the urinary excretion of glycocyamine during 24, later only 12, hours in which no protein was ingested with that on a day in which gelatin was ingested. Gelatin consists of about 25 per cent glycine and 9 per cent arginine (6). Each experiment ran for 2 days. On the evening preceding the 1st day the subject had a normal meal at 6 p.m. and 300 ml. of water in the course of the evening. During the following day he drank 1700 ml. of orange juice and an additional 300 ml. of water. This was the "non-protein" day in Fig. 1. Next day the subject drank 875 ml. of orange juice, and 1000 ml. of water containing 275 gm. of sucrose. He ingested

65 gm. of gelatin between 8 and 9 a.m. The gelatin contained 16 gm. of glycine and 5.8 gm. of arginine. This was the "gelatin" day.

A typical result is given in Fig. 1. It shows the rapid rise in the urinary excretion of glycocyamine after the ingestion of gela-
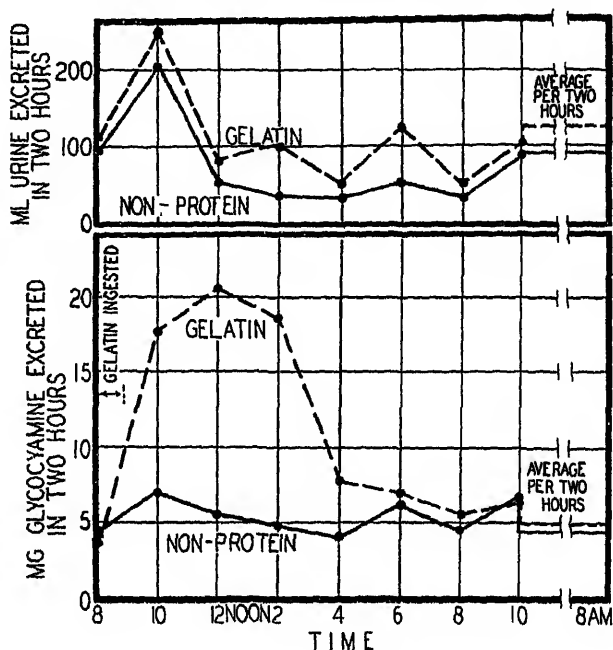
Fig. 1. Glycocyamine excretion in urine. The ordinates represent the glycocyamine excreted in the urine in the 2 hour period ending at the time indicated; the abscissae, the time at which the urine was voided. 65 gm. of gelatin were ingested between 8 and 9 a.m. The horizontal right-hand ends of both curves represent the total excretion in the night urine (10 p.m. to 8 a.m.) divided by 5.

tin. The maximum was attained between 10 a.m. and 12 noon. By 4 p.m. this rate had subsided near to the value on the non-protein day, and remained so during the following 14 hours. This rapid rise and fall in the rate of glycocyamine excretion occurred both when the arginine and glycine were taken in the form of gelatin and as the pure amino acids (see Figs. 1 and 2).

It is improbable that the increased urinary excretion of glyco-

cyamine observed after the ingestion of gelatin represents glyco-
cyamine "washed out" of the tissues.   If that had been the case,
the excretion during the last 14 hours of the gelatin day probably
would have fallen below that during the same interval on the non-
protein day; and the total amount of glycocyamine excreted over
the 24 hour period would have been nearly the same on these 2
days.   The 24 hour excretion of glycocyamine on the non-protein
day was 65 mg. and on the gelatin day 117 mg.   The lack of any
relation in Fig. 1 between urine volume and the amount of glyco-
cyamine excreted is in accord with this interpretation.

Table I is a summary of the findings in experiments similar to
that in Fig. 1 in five other subjects.   The data for the period
from 6 a.m. to 6 p.m. only are given.

TABLE I

Effect of Ingestion of 65 Gm. of Gelatin on Urinary Glycocyamine

Subject No.	Non-protein day		Gelatin day	
	Urine volume	Glycocyamine	Urine volume	Glycocyamine
	ml.	*mg*	*ml.*	*mg.*
1	475	43 7	890	87.5
2	1715	34.3	1785	114.0
3	370	34 0	600	60.0
4	1220	32 9	1520	88.2
5	470	24.4	1350	81.6

The figures for glycocyamine excretion on the non-protein days
in Table I indicate that over 24 hours they would have been of
the order of magnitude of 40 to 60 mg.   Weber (3) reported aver-
age figures for 24 hours of 39.5 mg. for males and 75.1 mg. for
females.   Weber's values are lower, probably because the figures
obtained with his analytical method represent only 75 per cent or
less of the glycocyamine present.

A series of experiments was carried out on two subjects in which
arginine and glycine, alone and together, were administered in
the form of the pure amino acids.   The two subjects remained
on the identical diet for the 9 days of the experiment.   Breakfast
and luncheon consisted of orange juice and coffee; dinner was a
normal meal in which the meat course was a weighed amount of
Hamburger steak.   During the day 200 ml. of water were drunk

every 2 hours.   Urine was collected every 2 hours from 6 a.m. to 6 p.m.   On the 3rd day of this régime the urine voided every 2 hours during the day was analyzed; the values obtained gave the "normal" graph in Fig. 2.   On the 5th day the subjects ingested 16 gm. of glycine between 8.30 and 9.30 a.m.; the glyco-
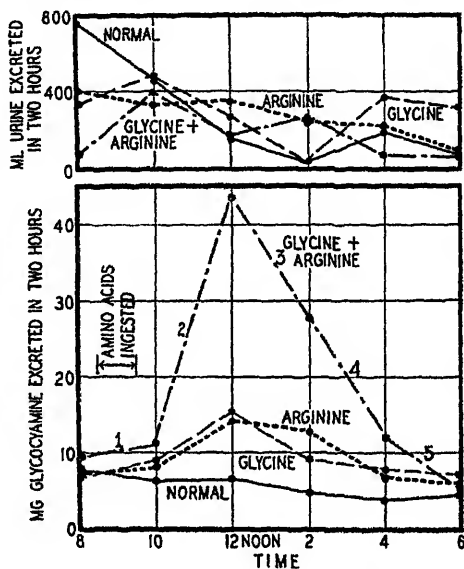
Fig. 2. Glycocyamine excretion in urine. The ordinates represent the glycocyamine excreted in the urine in the 2 hour period ending at the time indicated; the abscissae, the time at which the urine was voided. The amino acids were ingested, on each of the 4 days, in four portions between 8.30 and 9.30 a.m. The blood concentrations, at successive 2 hour intervals after the beginning of the experiment, on a day on which glycine and arginine were ingested together were *1*, 0.6, *2*, 0.4, *3*, 0.7, *4*, 0.8, and *5*, 1.0 mg. per cent.

cyamine excretion on this day is designated in Fig. 2 as "glycine." On the 7th day between 8.30 and 9.30 a.m. the subjects consumed 16 gm. of glycine and 4.8 gm. of arginine, and on the 9th day 4.8 gm. of arginine. The glycocyamine excretion on these days shown in Fig. 2 is designated "glycine + arginine" and "arginine."

Fig. 2 shows that after the ingestion of glycine alone more glycocyamine appeared in the urine. These increases were small,

however, compared to those obtained when the two amino acids were taken together. Thus on the normal day the glycocyamine excretion in 12 hours was 29.0 mg., on the glycine day 54.3 mg., on the arginine day 55.4 mg., and on the glycine + arginine day 99.0 mg. The sum of the increases over the normal day with glycine and arginine when these were ingested separately was 51.7 mg.; when ingested together it was 70 mg.

In the second subject the glycocyamine excretion on the normal day was 31.2 mg., on the glycine day 30.7 mg., on the arginine day 28.4 mg., and on the glycine + arginine day 61.8 mg.

The increase in glycocyamine in the urine after the ingestion of arginine and glycine was of the same order of magnitude as when the same amounts of these amino acids were ingested in the form of gelatin. The increase in urinary glycocyamine after the ingestion of gelatin may be ascribed to the arginine and glycine it contained.

These findings taken in conjunction with those obtained with surviving slices of kidney and with kidney extracts (4) may be considered as establishing the formation of glycocyamine by transamidination in man.

Blood was analyzed every 2 hours on the days on which the subjects ingested the glycine and arginine together. In the subject of Fig. 2 the concentrations were, in mg. per cent, 0.6, 0.4, 0.7, 0.8, and 1.0. The peak of the urinary excretion of glycocyamine occurred between the second and third of these values. There was no discernible relation between the glycocyamine concentration in the blood and its increased rate of excretion in the urine. The findings were essentially the same in the other subject.

We have made some preliminary observations on the excretion of glycocyamine by human subjects with kidney disease. On an ordinary diet they excreted less glycocyamine than normal individuals and the increase after the ingestion of arginine and glycine was less also. Thus in one subject who was diagnosed as having subacute glomerulonephritis the excretion of glycocyamine in the urine in an experiment similar to that of Fig. 1 was on the non-protein day 1.5, 3.0, 2.5, 2.6, 2.1, and 1.0 mg. in successive 2 hour intervals between 6 a.m. and 6 p.m.; and on the gelatin day 1.0, 3.1, 5.3, 2.4, 1.9, and 2.8 mg.

SUMMARY

1. When arginine and glycine are ingested together by human subjects, there is a rapid rise in the amount of glycocyamine excreted in the urine. This increase is greater than the sum of the increases which may occur when the same amounts of amino acids are taken separately.

2. A similar increase in glycocyamine excretion is observed after the ingestion of gelatin, which is rich in glycine and arginine. The order of magnitude of this increase is the same as that given by the quantity of arginine and glycine contained in the gelatin when these are administered as pure amino acids.

3. These findings indicate that in man glycocyamine is formed by transamidination.

BIBLIOGRAPHY

1. Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, **32**, 172 (1934).
2. Weber, C. J., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **109**, p. xcvi (1935).
3. Weber, C. J., *Proc. Am. Soc Biol Chem., J. Biol. Chem.*, **114**, p. cvii (1936).
4. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138**, 389 (1941).
5. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941)
6. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, American Chemical Society monograph series, New York (1929)

THE SHAPE OF PROTEIN MOLECULES*

II. VISCOSITY AND DIFFUSION STUDIES OF NATIVE PROTEINS†

By HANS NEURATH, GERALD R. COOPER, AND
JOHN O. ERICKSON

*(From the Department of Biochemistry, Duke University School of Medicine,
Durham, North Carolina)*

It is a well recognized fact that the diffusion constants of proteins are lower than is predicted by the theory for solutions of spherical, unhydrated molecules. It is also known that protein solutions exhibit a higher relative viscosity than is demanded by Einstein's viscosity equation (3) for rigid isolated spheres. These discrepancies between theory and experiment may be accounted for by assuming either that protein molecules are not spherical in shape, or that they are hydrated to an appreciable extent, or that both asymmetry and hydration exert a combined influence. An elucidation of this problem is of importance not only for defining protein molecules in terms of size, shape, and hydration but also for investigating the profound changes which occur in some or all of these properties incidental to protein denaturation.

Diffusion and viscosity are closely related physical phenomena. The relation between molecular shape and diffusion rate was treated hydrodynamically by Perrin (4) and by Herzog, Illig, and Kudar (5) for models having the shapes of prolate and oblate ellipsoids. The application of Perrin's theory in estimating the *apparent* shape of protein molecules, *hydration being neglected*, has been considered previously (1, 6). Attempts to correlate the viscosity of solutions of macromolecules with their shape have been undertaken frequently; however, a disconcerting disagree-

---

* For the first paper of this series see reference (1).

† Presented in part before the Division of Biological Chemistry at the One-hundredth meeting of the American Chemical Society at Detroit, September 9–13, 1940. A preliminary report has been published (2).

ment may be noted between the various equations that have been advanced.

For proteins, an empirical equation was suggested by Polson (7) which expresses the specific viscosity as a straight line function of the squares of the asymmetries as calculated from Perrin's diffusion equation.   Recently an equation based on an extension of the hydrodynamic theory of Jeffery (8) was derived by Simha (9), which, at present, appears to be the closest theoretical approach to the problem of the viscosity of molecules of asymmetrical shape.

The influence of hydration on the diffusion and viscosity of proteins (10, 11) is difficult to evaluate owing to a lack of information about the degree of hydration of these molecules.   However, certain deductions may be made on the basis of present information and these will be considered here.

The present paper describes quantitative viscosity and diffusion measurements of a number of well defined, native proteins.   While numerous viscosity measurements have been published in recent years (7, 12–16), the agreement between the data obtained by the various workers is far from satisfactory.   Most of these measurements were carried out in concentration higher' than about 1 per cent.   As sedimentation and diffusion data indicate a dependence of molecular kinetic properties of proteins on concentration (17, 18), emphasis was laid in the present investigation on confining the measurements to dilute solutions in which no interaction between the molecules may be assumed to occur.   An attempt is made in this paper to estimate the molecular shape and weight of the proteins from diffusion and viscosity data, and to correlate these findings with the results obtained by other physicochemical methods.   A similar analysis for denatured proteins will be presented in a publication to follow.

Theoretical

It would be beyond the scope of this paper to discuss in any detail the theories underlying the phenomenon of viscosity. Reference may be made to the excellent discussion by Burgers (19) and to the recent theoretical contributions by Guth, Eirich, Simha, and associates (20), Huggins (21), Peterlin (22), Robinson (23), and Simha (9).   In the following, attention will be drawn merely to various factors which require consideration for the problem under discussion.

In all cases in which the relation between the viscosity of a suspension and the shape of the suspended particles is considered, the following conditions are assumed to be fulfilled: (1) One is dealing with a strictly laminar flow, (2) the suspended particles are large and rigid and no slipping occurs, (3) the volume concentration of the suspension is small enough to exclude any interaction between the particles, and (4) in cases such as those considered here, the orientation of the particles is random.

1. It should be recalled that differences in the type of flow do exist between the streaming in a Couette apparatus on one hand and in capillary viscometers on the other. In the first case, the velocity increases linearly with the distance from the cylinder at rest, whereas in the second the velocity distribution is parabolic (24, 25). Viscosity theories are based on Couette streaming; however, they may also be applied to the results obtained with capillary viscometers if the solutions are dilute and the particles not too large (9, 26).

2. It is known that flexible or compressible particles will exhibit a lower viscosity than rigid ones (20, 21), and that the viscosity will also decrease if slipping occurs (19). Present information about protein structure provides evidence that protein molecules possess very definite size and shape, and there appears to be little doubt as to the rigidity of these molecules in the undenatured state. The hydrophilic character of their surface justifies the assumption that slipping does not occur.

3. The condition that the volume concentration be sufficiently small to avoid any interaction between the molecules has rarely been met with in the viscosity measurements that have been published. Theory predicts a direct proportionality between the specific viscosity, $\eta/\eta_0 - 1$, and the volume concentration, ϕ, the proportionality constant depending only on the shape of the solute molecules. Several empirical equations have been advanced (7, 13, 14, 27, 28) in order to describe the observed non-linear viscosity-concentration relation. Recently it has also been proposed to substitute the relative viscosity by its inverse function, the relative fluidity, and a linear relationship between the latter and the protein concentration has been shown to exist (29).

All these empirical equations, although of practical interest, are at present devoid of any theoretical basis. One is undoubtedly

on safer ground if one confines viscosity measurements to low protein concentrations, for which the theoretically demanded direct proportionality holds. The careful experiments by Bull (16), as well as those reported in this paper, verify this linear relation between specific viscosity and concentration.

4. Variations of the specific viscosity with the velocity gradient of flow may be caused by interaction between the particles, and by the orientation of asymmetric particles in the streaming liquid. The relation between the size and shape of the solute molecules, as expressed by their rotatory diffusion constant, and the velocity gradients at which the onset of structural viscosity due to orientation of the molecules may be observed has been discussed by Peterlin (22); an experimental verification for rod-shaped protein molecules has been reported recently by Robinson (23) and by Edsall and Mehl (30).

The present measurements were carried out with proteins which are neither large enough nor sufficiently asymmetric to exhibit this type of viscosity anomaly; values of k (the velocity gradient divided by the rotatory diffusion constant) are, unless the velocity gradients are extremely high, well within the region of complete Brownian motion. Indeed the relative viscosities of the proteins have been found to be independent of the velocity gradients, in agreement with the findings of Polson (7) and Bull (16).[1]

The proportionality constant between specific viscosity and volume concentration, K, will be higher than 2.5 if the particles deviate from strictly spherical shape. Theoretically, any irregularity in shape will produce an increase in K; however, only certain geometrical models have been considered by the hydrodynamic theory, and while only these particular models can be employed for an interpretation of the viscosity data there is no absolute evidence that proteins actually do possess these particular shapes.

Polson (7) has pointed out that neither Burger's (19) nor Kuhn's (31) equation was applicable to proteins. From viscosity measurements of a number of proteins in concentrations between 1 and 5 per cent Polson arrived at an empirical viscosity equation which

[1] Nothing is known at present about the viscosity of protein solutions in the lowest region of velocity gradients; an investigation of viscosity anomalies in regions of extremely low gradients is under way in this laboratory.

differs from the Kuhn equation in the choice of the numerical constants.   According to Polson,

$$\eta_{sp} = 4.0 + 0.098(b/a)^2 \tag{1}$$

where b/a is the ratio of long to short axis of a prolate ellipsoid.
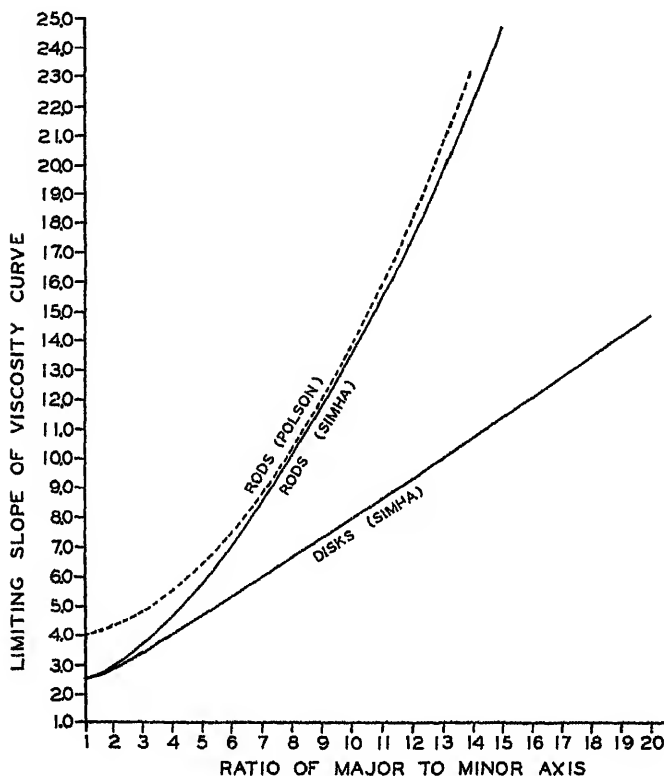
FIG 1. Relation between viscosity and the axial ratio of protein molecules according to Simha and Polson.   The upper two curves refer to the viscosity equations of Polson and Simha for molecules having the shape of prolate ellipsoids (rods); the lower curve to Simha's equation when solved for the shape of oblate ellipsoids (disks).

A graphical comparison between Polson's equation and that of Simha[2] (9), which is based on strictly hydrodynamic considerations and takes account of Brownian motion, is made in Fig. 1.

[2] In applying Simha's equation to our data we have used it in its original form (Equation 6 of reference (9)) and solved it graphically for given values of the axial ratio.   (See also (32).)

Polson's equation predicts, for a given specific viscosity, lower asymmetries than Simha's equation. The discrepancies are largest in regions of low and very high asymmetries, and small for intermediate asymmetries.

Substitution of values for a/b obtained from viscosity measurements into Perrin's diffusion equation (4) should yield dissymmetry constants, f/f_0, identical with those determined directly from molecular weight and diffusion measurements.

$$D(MV)^{\frac{1}{3}} \cdot (f/f_0) = K' \tag{2}$$

where K' is a constant for a given temperature, M the molecular weight, V the partial specific volume, and D the diffusion constant.

Finally it should be possible to calculate molecular weights from viscosity and diffusion data by combining f/f_0 computed from viscosity data with the observed value for the diffusion constant.

$$M = \frac{R^3 T^3}{162\pi^2 \eta_0^3 N^2} \cdot \frac{(f_0/f)^3}{D^3 V} \tag{3}$$

where T is the temperature, η_0 the viscosity of the solvent, N Avogadro's number, and the other symbols of the meaning given above. Such a method of determining the molecular weight has, however, the disadvantage that both diffusion and dissymmetry constants enter into the equation as their respective third powers, thus strongly magnifying any experimental error.

Measurements

Viscosity—Viscosity measurements were performed in Ostwald viscometers with the modifications of Jones and Fornwalt (33), to reduce the influence of surface tension and kinetic energy.   Fig. 2 shows diagrammatically the essential details of construction of the viscometers.   Four such instruments were used, the only differences between them being the volume of the drainage bulb and the time of flow.   The viscometers were clamped rigidly, by means of a slot and clasp arrangement, in a metal frame which was mounted firmly inside the water bath.   Observations of the meniscus marks were made through a cathetometer.   The time of flow was measured with an electric Cenco-Harrington timer capable of being read to 0.01 second.   It was frequently checked against a high precision stop-watch.

Table I gives the dimensions and physical constants of the four viscometers. The radius of the capillary, r, was measured with
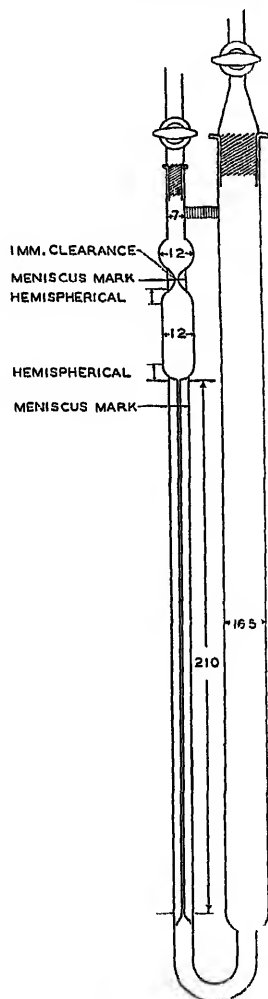
FIG. 2. Diagram of the capillary viscometer

mercury prior to the construction of the viscometer. The radius of the reservoir tube, R, was large enough to minimize any error arising from accidental variations in the working volume.

The cylindrical shape of the reservoir tube allowed the maximum velocity gradient to be varied over a wide range by filling the viscometers with varying amounts of solution. Values for the maximum velocity gradient, $G_{max.}$, produced when a working volume of 10, 20, and 30 cc., respectively, was employed, are included in Table I. They were calculated (24) with the equation

$$G_{max} = 4\omega/r \tag{4}$$

where ω, the mean velocity, is

$$\omega = V/r^2\pi t \tag{5}$$

r is the radius of the capillary, V the volume of the drainage bulb, and t the time in seconds.

TABLE I

Physical Constants of Capillary Viscometers

r = radius of capillary, l = length of capillary, R = radius of reservoir tube, V = volume of drainage bulb, $G_{max.}$ = maximum velocity gradient for a working volume of 10, 20, and 30 cc. of water respectively.

Vis- com- eter No.	r	l	R	V	G_{max}		
					10 cc.	20 cc.	30 cc.
	cm.	cm.	cm.	cc.	sec.$^{-1}$	sec.$^{-1}$	sec.$^{-1}$
1	0 0303	21.3	0 80	6.885	1730	1320	880
2	0.0306	21.0	0 83	5 881	1710	1290	890
3	0.0297	21.0	0 83	3.422	1470	1090	700
4	0.0306	21.0	0.87	5 567	1700		

Comparison of the mean velocity of flow with the limiting velocities calculated by Grueneisen ((24) p. 112) established that in our viscometers retardations in flow due to deviations from Poiseuille's law did not exceed 0.1 to 0.2 per cent.

The viscometers were filled by inserting a calibrated 10 cc. pipette into the reservoir tube. The water constants of the viscometers were redetermined frequently and were found not to vary more than 0.1 second during a period of about 3 weeks. The time of flow was determined with an accuracy of ±0.06 second. Measurements for which in five to eight successive runs the time varied beyond this limit were discarded. All measurements were carried out at 25° ± 0.01°.

Buffer and protein solutions were freed from contaminations prior to each viscosity determination by filtering through sintered glass crucibles. Protein concentration was determined by dry weight and Kjeldahl analyses of the stock solutions and by Kjeldahl analysis of the dilutions prepared therefrom.

Diffusion—Diffusion measurements were carried out with the refractometric scale method (34) as described previously (15, 35). Protein solutions of the desired concentrations were mixed with buffer and salt components of the same composition and concentration. Dialysis was usually extended over a period of 3 days, at 5°, and the outside buffer solution replaced with fresh stock solution every 24 hours.

Density—Density measurements were carried out in pycnometers of about 5 cc. volume at 25°. The density was measured in duplicates at a few protein concentrations and the density for any other protein concentration determined by interpolation.

Material

Lactoglobulin—Crystalline lactoglobulin (Palmer) was obtained through the courtesy of Dr. R. K. Cannan.[3] The crystals were dissolved in 0.1 N sodium chloride, any insoluble denatured material filtered off, and recrystallization carried out by dialyzing the protein solution against distilled water at 5°.

Pepsin—Two crystalline pepsin fractions were prepared[4] from Parke, Davis (1:10,000) material, according to the method of Philpot (36) with the refinements as introduced by Steinhardt (37). The two fractions were separated from each other during the second crystallization; the more insoluble fraction (Pepsin A) crystallized after the pH of the 0.105 N acetate buffer had been lowered from 4.7 to 3.6, while the more soluble fraction (Pepsin B) was obtained by acidifying the mother liquor of Pepsin A to pH 3.3. The crystals were stored under magnesium sulfate solution.

Before each experiment, the crystals were washed three times with 10 to 20 cc. of 0.002 N HCl (pH 2.7) per gm. of filter cake. They were then dissolved in the proper amount of acetate buffer

[3] We are greatly indebted to Dr Cannan for supplying us with this material.

[4] We are indebted to Dr. P. H. Handler for his assistance in the preparation of pepsin

of pH 5 and dialyzed against successive 200 cc. portions of acetate buffer of the same composition and pH.

Pseudoglobulins—The fractionation of the serum globulins was effected by a combination of fractional precipitation with ammonium sulfate with the method of isoelectric salt-free precipitation as described by Green (38). 4000 cc. of filtered, fresh horse serum, diluted with an equal volume of 0.8 M ammonium sulfate solution, were adjusted to pH 6.4 and the salt concentration gradually increased to 1.36 M ammonium sulfate, with the rotating membrane method of McMeekin (39). After standing overnight, a large bulky precipitate, GI, was filtered off. A second globulin fraction precipitated from the filtrate after the ammonium sulfate concentration was raised to 1.6 M. This fraction, GII, was filtered off and a third fraction, GIII, was obtained in a similar manner by increasing the salt concentration to 2.1 M. The fractions, GI, GII, and GIII, were purified by dissolving them in a minimum amount of water, filtering off any insoluble residue, and diluting with 1 M ammonium sulfate solution to one-fifth of the original volume of serum. Since in the first stages of precipitation, coprecipitation of the various globulins may be expected to occur, the solutions of GI, GII, and GIII were then adjusted to an ammonium sulfate concentration at which the preceding less soluble fraction precipitates; thus the salt concentration of GI was raised to 1 M, at pH 6.4, to precipitate any denatured material, that of GII to 1.36 M, to precipitate occluded GI, and that of GIII to 1.6 M, to eliminate any coprecipitated GII. These precipitates were discarded and the ammonium sulfate concentration of the filtrates slowly raised to 1.36, 1.6, and 2.1 M for GI, GII, and GIII respectively. A third precipitation of these three fractions was carried out in the same manner, again at pH 6.4 and at a protein concentration of 2 per cent. The water-insoluble euglobulins were separated at this stage from the main globulin fractions by the method of Green (38). The precipitates of GI, GII, and GIII were dissolved in water and dialyzed in the cold until free of ammonia. The protein solutions were then adjusted to pH 7.1, any insoluble material filtered off, and the euglobulin fractions PI, PII, and PIII precipitated by proper pH adjustment (to pH 6.2 and 5.0 respectively). The filtrates, containing the pseudoglobulins, were collected separately for GI,

GII, and GIII, and the corresponding pseudoglobulins GI, GII, and GIII precipitated by adjustment of the ammonium sulfate concentrations to 1.36, 1.6, and 2.1 M respectively at pH 6.4, as described before. The precipitates were dissolved in water, again dialyzed, and after removal of any undissolved material concentrated and stored.

These fractions, free from any isoelectrically precipitable euglobulins, differed from one another chemically in respect to solubility in ammonium sulfate and carbohydrate content. The concentrated solutions of GI and GII were colorless, that of GIII slightly bluish green. The nitrogen content, as determined by the Kjeldahl method and dry weight analysis, was 15.6 per cent in all three cases. The carbohydrate content, as determined by the orcinol method of Sørensen and Haugaard (40) with blank correction for the color developed by sulfuric acid and protein, was 0.76, 1.03, and 1.38 mg. per 100 mg. of protein for GI, GII, and GIII, respectively. These figures refer to a mixture of equal parts of dextrose and mannose as standard.

Serum Albumin—A number of methods are now available for preparing crystalline fractions of serum albumin (39, 41–43). Measurements on preparations obtained according to the various procedures were carried out to prove their similarity or else to indicate the superiority of one method over another.

Serum Albumin (Hewitt), SA_I—Hewitt's method (41) has been modified in that the salt concentration was increased stepwise, within narrow limits, in order to attain a more quantitative separation of the various fractions, depending on differences in solubility. The pH of the supernatant protein solution, resulting from the first precipitation of the globulin fractions containing 2.1 M ammonium sulfate at pH 6.4, was lowered to 5.2 by the addition of acetic acid, and the first crop of crystals separated at this salt concentration (SA_A). Upon increasing the ammonium sulfate concentration of the supernatant solution, with the rotating membrane method, a second fraction precipitated at 2.2 M; it was partly crystalline (long needles) and partly amorphous (SA_B). Only a small precipitate appeared at 2.3 M ammonium sulfate (SA_C), but a heavy strongly pigmented precipitate appeared after the salt concentration had been raised to 2.5 M (SA_D). Finally a small amount of straw-colored precipitate (SA_E) was obtained

after more ammonium sulfate was added to the filtrate of SA_D.[5]
Each fraction obtained in the above manner was reprecipitated
several times.

The SA_A fraction which crystallized between 1.9 and 2.1 M
ammonium sulfate at pH 5.2 was subjected to one more crystalliza-
tion, at pH 4.8.   The protein solution was then adjusted to pH 7
and half saturated with ammonium sulfate.   A small amount of
amorphous materials which precipitated was filtered off; the
protein contained in the supernatant liquid was subjected further
to three crystallizations at pH 5.5 with the protein concentration
adjusted to about 2 per cent.   By increasing the ammonium
sulfate concentrations in steps of 0.1 M it was possible to separate
the more soluble from the more insoluble fractions. SA_I con-
tained the more soluble fractions which crystallized between 2.3
and 2.5 M ammonium sulfate at pH 5.5.   Altogether seven
crystallizations were carried out before this material was used for
diffusion and viscosity experiments.   The nitrogen content was
15.1 per cent.

Seroglycoid (SA_D)—The albumin fraction used for the prepara-
tion of SA_D was collected between 2.4 and 2.5 M ammonium sulfate
at pH 5.2.   The strongly pigmented precipitate was dissolved in
a volume of water equal to one-eighth of the original volume of
serum and recollected at the same salt concentration and pH.
A third reprecipitation was carried out in a similar manner, and
the precipitate dissolved in water, dialyzed, and stored.   The
solution had a brown color; the carbohydrate content was about
4.4 mg. per gm. of protein, the nitrogen content 14.6 per cent.
It is interesting to note that the purified material was, contrary to
Hewitt's (41) findings, of low carbohydrate content, a result in
agreement with the data of Rimington and Van den Ende (43).

Serum Albumin (McMeekin) (SA_α)—This preparation was ob-
tained by following the procedure described by McMeekin (39).
The first crop of crystals was collected between 2.1 and 2.48 M
ammonium sulfate at pH 6.4 and was recrystallized once in the
same manner.   The third crystallization was carried out in a
solution of about 10 per cent protein; here precipitation occurred

---

[5] SA_D and SA_E are probably identical with the fractions which Riming-
ton and Van den Ende (43) described as Sg-66 and Sg-75 in a publication
which appeared while this paper was in preparation.

after the ammonium sulfate concentration had been raised to 2.0 M at pH 5.5. In the fourth crystallization, fractional precipitation was employed by collecting separately the crystals coming down at 1.6 and 1.9 M ammonium sulfate respectively at pH 5.5. The less soluble fraction was concentrated to 25 per cent in the presence of sulfuric acid of pH 4.0 and yielded the type of crystals described by McMeekin. They were subjected to one more crystallization in concentrated solution at pH 5.2 at 1.6 M ammonium sulfate (SA_α). No water-insoluble, crystalline protein sulfate could be obtained from the more soluble fraction which, in the fourth crystallization, precipitated at 1.9 M ammonium sulfate. This finding was observed with three batches of fresh horse serum and indicates that the formation of protein crystals insoluble in water under the conditions described by McMeekin is confined to the least soluble fraction of crystalline horse serum albumin.

Serum Albumin (*Kekwick*)—Two crystalline serum albumin fractions were prepared according to Kekwick (42) with sodium sulfate as the precipitating agent. SA_B was, after three crystallizations, practically free of carbohydrate (carbohydrate-nitrogen ratio 0.08), whereas the more soluble fraction, SA_A, contained after three crystallizations 1.95 per cent carbohydrate, in agreement with Kekwick's data.

Results

Viscosity—In Table II the results of viscosity measurements are given. The relative viscosity was found to be a linear function of the protein concentration up to at least 11 mg. per cc.

Comparison of our data with those of Polson (7) is possible only for lactoglobulin, pepsin, and crystalline serum albumin, at a protein concentration of 1 per cent. For lactoglobulin Polson finds a somewhat higher value, whereas our Pepsin B gives the same value as the unfractionated crystalline preparation used by Polson. In the case of serum albumin Polson finds a considerably higher value than was found for our monodisperse crystalline fractions.

In Table III, values for the relative viscosity of three proteins measured at three different velocity gradients are listed. The slight variations in η/η_0 are probably without significance and

TABLE II
Viscosities of Proteins

Protein	Concentration	$\frac{\eta}{\eta_0}$	Protein	Concentration	$\frac{\eta}{\eta_0}$
	per cent			*per cent*	
Pepsin A (least soluble fraction). Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.15	1.006_1	Serum albumin (Kekwick) SA$_B$. Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.225	1.009_0
	0.23	1.009_5		0.445	1.019_1
	0.35	1.016_3		0.670	1.029_2
	0.52	1.023_3		0.84	1.036_7
	0.69	1.031_7		1.09	1.046_8
	0.92	$1\ 041_7$		1.34	1.057_6
	1.165	1.052_8	Serum albumin (seroglycoid) SA$_D$. Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0 15	1.008_0
	1.555	$1\ 072_2$		0.265	1.013_9
Pepsin B (soluble fraction). Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.255	1.010_4		0.535	1.025_8
	0.51	1.019_6		0.90	1.047_0
	0.61	1.024_3		1 54	1.078_4
	1 015	1.039_2		1.71	1.088_0
	1.035	1.040_4	Pseudoglobulin GI. Solvent, 0.05 M acetate buffer, 0.2 M NaCl; pH 5.5	0.28	1.020_1
	1.38	1.054_0		0.43	1.030_9
	1.63	1.066_9		0.57	1.041_0
Lactoglobulin. pH 5.0; solvent, 0.05 M acetate buffer, 0.1 M NaCl	0.195	1.007_4		0.86	1.061_9
	0.355	1.015_6		1.13	1.083_0
	0.490	1.020_6		1.38	1.102_8
	0.765	1.031_6		1.72	1.128_3
	1.775	1.076_3	Pseudoglobulin GII. Solvent, 0.05 M acetate buffer, 0.2 M NaCl; pH 5.5	0.30	1.020_8
	1.972	1.084_2		0 44	1.029_5
pH 7.0; solvent, 0.03 M phosphate buffer, 0.1 M NaCl	0.95	1.040_7		0.50	1.040_4
	1.28	1.053_5		0.77	1.051_1
	1.70	1.071_2		0.99	1.065_2
Serum albumin (Hewitt) SA$_I$. Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.21	1.008_9		1.195	1.079_7
	0.42	1.018_5		1.28	1.085_4
	0.525	1.021_8		1.39	1.092_5
	0.63	1.026_6		1.65	1.110_6
	0.84	1.037_1		2.21	1.153_5
	1.05	1.045_8	Pseudoglobulin G-III. Solvent, 0.05 M acetate buffer, 0.2 M NaCl; pH 5.5	0.25	1.014_2
	1.165	1.049_3		0.50	1.030_4
	1.40	1.060_4		0.68	1.041_4
Serum albumin (McMeekin) SA$_\alpha$. Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.20	1.007_4		0.94	1.058_8
	0.40	1.015_5		1.14	1.071_6
	0.455	1.016_8		1.25	1.080_3
	0.86	1.032_5		1.67	1.109_8
	1.035	1.039_0			
	1.28	1.048_2			
Serum albumin (Kekwick) SA$_A$. Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0	0.195	1.007_9			
	0.385	1.017_1			
	0.575	1.024_1			
	0.795	1.034_1			
	0.98	1.042_2			
	1.195	1.052_2			
	1.47	1.066_1			

within the limits of the experimental error of these particular measurements.

Diffusion—Diffusion constants were calculated from the curves obtained when, in the usual manner, scale line displacements were plotted against the positions of the displaced lines. The following

TABLE III

Relation between Velocity Gradient and Relative Viscosity

Protein	Concentration	G^0_{max}	$\frac{\eta}{\eta_0}$
	per cent	*sec.*$^{-1}$	
Lactoglobulin	1.97	1470	1.084
		1090	1.085
		700	1.086
Pseudoglobulin GI	1.72	1730	1.128
		1320	1.126
		880	1.128
" GIII	1.67	1470	1.110
		1090	1.112
		700	1 110

$G^0_{max.}$ = maximum velocity gradient prevailing when the viscometers were filled with 10, 20, and 30 cc. of water respectively.

equations were employed for the computation of the diffusion constants (34).

$$D_1 = \frac{\mu^2}{2t}\left(\frac{l-b}{l}\right)^2 \frac{1}{G^2} \tag{6}$$

$$D_2 = \frac{A^2}{H_m^2 \, 4\pi t}\left(\frac{l-b}{l}\right)^2 \frac{1}{G^2} \tag{7}$$

$$D_3 = \frac{x_1^2 - x_2^2}{4t \ln (H_2/H_1)}\left(\frac{l-b}{l}\right)^2 \frac{1}{G^2} \tag{8}$$

Here D is the diffusion constant in sq. cm. per second (uncorrected for the viscosity of the solvent), t the time in seconds, $(l-b)/l$ a photographic factor, G the optical magnification, μ half the distance between the inflection points of the diffusion curves, A the area under the curves, and H_m their maximum height. x_1 and x_2 are the abscissae of points on the curves of ordinates H_1 and H_2.

TABLE IV

Diffusion Constants of Proteins

t = time in seconds, D = measured diffusion constant in sq.cm. per second, D' = average diffusion constant corrected for the viscosity of the solvent. Solvents, for pepsin and serum albumin fractions 0.023 M acetate buffer, 0.2 M NaCl, pH 5.0; for the pseudoglobulins, 0.05 M acetate buffer, 0.2 M NaCl, pH 5.5.

Pepsin A (least soluble fraction)					Serum albumin (seroglycoid) SA_D				
Concentration	t	D_1	D_2	D_3	Concentration	t	D_1	D_2	D_3
per cent	sec.	10^{-7}	10^{-7}	10^{-7}	per cent	sec.	10^{-7}	10^{-7}	10^{-7}
1.0	14,480	9.03	9 00		1 0	45,480	6.67		6.45
	23,880	8.79	9 27	8.91		68,400	6 68		7.08
	35,880	8.67	9 06	9.16		91,800	6.80		7 08
	77,100	8 79	9.25	9 05	0.5	20,520	6 45		
						38,880	6.80		
Average		8 95 × 10^{-7} ± 0.22			Average		6 67 × 10^{-7} ± 0 24		
D'		9.17 × 10^{-7}			D'		6 93 × 10^{-7}		

Pepsin B (soluble fraction)				
0 7	12,600	9 76	9.75	10.1
	23,760	9.76	9 32	9 95
	41,100	10.1	9.24	
	78,120	9 58		
Average		9 72 × 10^{-7} ± 0.31		
D'		9 96 × 10^{-7}		

Pseudoglobulin GI				
1 5	37,200	4.39	4.19	4.27
	69,600	4.30	4.08	
0 7	15,840	4.48	4.08	
	32,040	4.60	4.19	4.35
	44,640	4.56	4 38	4.48
	81,660	4.52		4.45
0.5	37,320	4.69	4.35	4.27
	75,660		4 29	4.61
Average		4.43 × 10^{-7} ± 0 17		
D'		4.61 × 10^{-7}		

Serum albumin (Hewitt) SA_I				
0.7	10,680	7.26	7 12	6.90
	28,380	6.84	6 55	6.86
	47,580	6.75	6.33	6.49
	85,960	6 79	6.05	6.52
Average		6.78 × 10^{-7} ± 0.27		
D'		6 95 × 10^{-7}		

Pseudoglobulin GII				
1.5	43,500	4 26	4.29	4 53
	65,400	4 38	4.30	4 44
1.0	43,200	4 72	4.63	
	73,800		4.75	4.62
0.7	21,750	4.51	4.53	4.63
	44,430	4.49	4.71	4.72
	78,630	4.38	4.62	
0.5	19,440		4.44	
	35,640	4.83	4.52	
Average		4.59 × 10^{-7} ± 0.08		
D'		4.75 × 10^{-7}		

Serum albumin (McMeekin) SA_α			
1 0	18,540	7 03	7.35
	33,420	7.29	7.33
	60,960	7.10	7.21
	84,900	7.24	7.12
0.7	20,400	7.03	7.29
	47,220	7.12	7.09
Average		7.18 × 10^{-7} ± 0 15	
D'		7.36 × 10^{-7}	

TABLE IV—*Concluded*

Pseudoglobulin GIII				
Concen-tration	*t*	D_1	D_2	D_3
per cent	*sec.*	10^{-7}	10^{-7}	10^{-7}
1.5	43,200	4.14	4.17	
	64,800	4.11	4.29	4.14
1.0	54,000	4.38	4.42	4.34
	76,860	4.40	4 51	4.52
0.7	37,620	4.29	4.06	4.26
	77,340	4.35	4.00	4.20
0.5	23,040			4 75
	35,640	4.45		4.41
Average .		$4.36 \times 10^{-7} \pm 0.18$		
D'		4.55×10^{-7}		

In Table IV the results of the diffusion experiments are given. D_1, D_2, and D_3 are the diffusion constants calculated with Equations 6, 7, and 8 respectively. Each D_3 value listed is the mean of at least three values, determined from various parts of the diffusion curves. Unless otherwise stated, the deviations from the mean never exceeded, and were usually less than, the standard deviation observed for all values of a given experiment. D' is the diffusion constant corrected for the viscosity of the solvent, according to the equation (34)

$$D' = D \, \eta/\eta_0 \qquad (9)$$

DISCUSSION

From the slope of the linear part of the curves obtained when the specific viscosity is plotted against the volume concentration, the molecular asymmetries have been calculated, with Polson's and Simha's equations. The partial specific volume of the anhydrous protein has been used for calculating the volume concentration, and, therefore, the asymmetries refer to *unhydrated* protein molecules. The results are listed in Table V.

Attention may be drawn to the lower viscosity of serum albumin SA_α (McMeekin), compared with fractions SA_I (Hewitt) and SA_A and SA_B (Kekwick). This difference in relative viscosity is, however, compensated by differences in the diffusion constants,

thus giving the same molecular weight for these four preparations. Similar differences have been found for the less soluble (Pepsin A) and the more soluble fraction (Pepsin B) of pepsin.

Comparison between the molecular shape computed on one hand from viscosity data and on the other from diffusion and sedimentation data may be best made on the basis of the dissymmetry constants and molecular weights, calculated with Perrin's equa-

TABLE V

Apparent Asymmetries of Proteins (Hydration Being Neglected) Calculated from Viscosity Data with Polson's and Simha's Equations

η_{sp}/cV_0 = limiting slope of specific viscosity-volume concentration curves.

Protein	$\frac{\eta_{sp}}{cV_0}$	Ratio of major to minor axis		
		Rods		Disks
		Polson	Simha	Simha
Pepsin A (least soluble fraction) . .	6.00	4.5	5.2	7.0
" B (soluble fraction).	5.25	3.6	4.6	5.9
Lactoglobulin....	5.60	4.1	4.9	6.4
Serum albumin (Kekwick) SA$_A$ ⎫ SA$_B$ ⎭ .	5.75	4.3	5.0	6.7
" " " (Hewitt) SA$_I$	5.70	4.2	4.9	6.6
" " (McMeekin) SA$_\alpha$... ..	5.05	3.3	4.4	5.6
Seroglycoid SA$_D$..	6.90	5.4	5.9	8.3
Pseudoglobulin GI	9.65	7.6	7.7	12.4
" GII..	8.80	7.0	7.2	11.1
" GIII..	8.25	6.6	6.8	10.3

tions and Equation 3. The results obtained are listed in Table VI. Column 2 refers to the observed diffusion constants, Column 3 to the slope of the viscosity curves. Columns 4 and 5 list the dissymmetry constants and molecular weights as obtained from diffusion and sedimentation data. The following columns (Nos. 6 to 11) list values for these constants as calculated from viscosity, and viscosity and diffusion data respectively, for the models of prolate (rods) and oblate (disks) ellipsoids.

In the case of egg albumin and the pepsin fractions Polson's equation appears to yield asymmetries fairly consistent with those

TABLE VI

Dissymmetry Constants and Molecular Weights of Proteins from (a) Diffusion and Sedimentation* and (b) Diffusion and Viscosity Data

D^{25} = diffusion constant, η_{sp}/cV_0 = limiting slope of specific viscosity-volume concentration curves, f/f_0 = dissymmetry constant, M = molecular weight.

			Diffusion and sedimentation		Viscosity					
					Rods				Disks	
					Polson		Simha		Simha	
Protein	D^{25}	$\dfrac{\eta_{sp}}{cV_0}$	$\dfrac{f}{f_0}$	M	$\dfrac{f}{f_0}$	M	$\dfrac{f}{f_0}$	M	$\dfrac{f}{f_0}$	M
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	10^{-7}									
Pepsin A	9.17	6.00	1.13†	35,500†	1.22_0	34,400	1.26_4	30,900	1.32_4	26,800
" B	9.96	5.23			1.15_2	31,800	1.22_0	26,800	1.27_0	23,800
Egg albumin, §	8.86	5.13	1.16	44,000	1.14_0	46,700	1.21_4	39,000	1.26_4	34,400
Lactoglobulin‡	8.33	5.60	1.25	41,800	1.18_6	49,800	1.24_4	43,200	1.29_6	38,200
Serum albumin (Kekwick)‖ SA_A ‖ SA_B	6.99	5.73	1.27	70,400	1.20_0	81,600	1.25_2	71,900	1.31_0	62,700
" " (Hewitt) SA_I	6.95	5.71	1.25	70,000	1.19_7	83,800	1.25_3	73,200	1.30_6	64,500
" " (McMeekin) SA_α	7.36	5.07			1.13_0	83,900	1.20_7	68,800	1.25_4	61,400
" " (seroglyoid)SA_D	6.93	6.91			1.28_0	69,200	1.30_4	65,400	1.38_6	54,500
Pseudoglobulin GI	4.61	9.63	1.44†	167,000†	1.40_6	177,800	1.41_5	174,100	1.55_0	132,500
" GII	4.75	8.80			1.37_0	174,700	1.38_5	170,100	1.50_0	133,100
" GIII	4.55	8.27			1.34_5	210,700	1.36_2	203,400	1.47_0	161,700

* Values taken from reference (18).
† Values for unfractionated material.
‡ Diffusion constant determined by Polson (17).
§ Viscosity data by Bull (16).
‖ Diffusion constant determined by Kekwick (42).

calculated from diffusion and sedimentation data.[6] With all other proteins studied, however, this empirical equation fails in that the dissymmetry constants calculated with its aid are considerably lower, and molecular weights considerably higher, than those observed. It may be seen from the data obtained for lactoglobulin, the crystalline serum albumin fractions, and the pseudoglobulins, that Simha's equation is satisfactory for calculating apparent molecular shapes, and in combination with diffusion data, molecular weights of anhydrous proteins. The assumption of proteins resembling in shape oblate ellipsoids gives results incompatible with those obtained from sedimentation and diffusion measurements.

In the preceding considerations, the influence of hydration on molecular asymmetry has, as a first approximation, been neglected. It may readily be seen, however, that any appreciable extent of hydration influences the results obtained from either diffusion or viscosity data. The differences between the measured diffusion constant (D) and that calculated for a spherical unhydrated molecule (D_0), previously ascribed to molecular asymmetry, may, theoretically, also be ascribed to effects of hydration (11, 32, 44). Similarly, one may conceive that the deviations of the slope of the viscosity curves from the theoretical value of 2.5 are due to the fact that the volume concentration of the *anhydrous* protein (cV_0) has been plotted against the specific viscosity, instead of the volume concentration of the *hydrated* protein (cV_h) (45) being plotted. The relation between the degree of hydration and the partial specific volume of the hydrated protein can, assuming volume additivity, be expressed as (44)

$$V_h = (r + 1)V_{10} = rV_1 + V_0 \qquad (10)$$

where V_0 is the partial specific volume of the anhydrous protein, V_1 that of water, and r the number of gm. of water combined with 1 gm. of anhydrous protein. V_{10} is the partial specific volume of the hydrated protein (per gm. of hydrate) and V_h the apparent specific volume of the hydrated protein (per gm. of anhydrous

---

[6] Even in the case of pepsin Polson's equation may not be applicable. The reference values for the dissymmetry constant and molecular weight were obtained from measurements on unfractionated, crystalline material (17, 36) and may be subject to revision.

protein), sometimes referred to as specific hydrodynamic volume (11, 45). Assuming various degrees of hydration (r), values for the axial ratio have been computed for a few representative proteins with the Simha equation (Table VII).

Inspection of Table VII shows a decrease of the apparent asymmetries as hydration increases. This decrease is, however, greater for the less asymmetric molecules than for the more asymmetric ones. Thus the asymmetry decreases for Pepsin B from 4.6 (assuming prolate ellipsoids) at zero hydration, to about

TABLE VII

Influence of Hydration on Asymmetries of Proteins, from Viscosity Data

Hydration	Pepsin B		Lactoglobulin		Serum albumin (Hewitt and Kekwick)		Pseudoglobulin GII	
	Ratio of major to minor axis							
	Rods	Disks	Rods	Disks	Rods	Disks	Rods	Disks
per cent								
0	4 6	5.9	4.9	6.4	4.9	6.6	7.2	11.1
10	4 0	4.9	4.3	5.4	4.4	5.6	6.5	9.5
25	3.3	3.9	3.6	4.3	3.6	4.4	5.6	7.8
33	**3.0**	3 4	**3.2**	3.8	**3.3**	3.9	**5.2**	7.1
50	2.3	2.6	2.6	2.9	2.7	3.1	4.6	5.9
75	1.4	1.4	1.8	1.9	1.9	2.0	3.7	4.5
100							3.1	3.6
	1 (82%)		1 (94%)		1 (96%)		1 (188%)	

The values in bold-faced type represent, as discussed in the text, the most probable values for the true asymmetries (assuming 33 per cent hydration).

one-third of this value at 75 per cent hydration. For the more asymmetric pseudoglobulin GII, the corresponding change is from 7.2 to only one-half of this value. Obviously, for highly asymmetric molecules, such as denatured proteins (15), the correction for hydration will be small. With the last value of each group of Table VII there is given, in parentheses, a value for the amount of hydration which, on the basis of Simha's equation, would have to be assumed in order to reduce the apparent asymmetries to unity (spherical shape).[7]

[7] According to Polson's equation, the corresponding values are considerably lower, especially for proteins of low apparent asymmetry. Thus, for

Measurements of the degree of hydration of proteins carried out by various workers (10, 46, 47) point to a value of about 33 per cent. Using this value, one calculates with Simha's equation the asymmetries printed in bold-faced type in Table VII. It is the authors' opinion that these values are the closest approximations to the true molecular shapes.

Comparative Observations on Some Physicochemical Properties of Serum Proteins

In the following, attention is drawn to some chemical and physical properties of the serum protein fractions which have been prepared.

Purification of the pseudoglobulins GI, GII, and GIII according to the method described in this paper yields fractions of a very high degree of monodispersity. The standard deviation of the diffusion constants is small and within the experimental error generally involved in diffusion measurements. Our values for GI and GII are about the same as those found by Tiselius (48) for the electrophoretically isolated globulins, α, β, and γ ($D^{25} = 4.7 \times 10^{-7}$), while the value for GIII is somewhat lower. The antidiphtheria globulin investigated by Pappenheimer, Lundgren, and Williams had a diffusion constant of $D^{25} = 4.4 \times 10^{-7}$ (49). Values reported in Table IV have been duplicated with material prepared in the same manner from another batch of normal horse serum.

Two of the pseudoglobulins, GI and GII, have identical molecular weights, while that of GIII is considerably higher.[8] The apparent asymmetries of these three proteins decrease with increasing solubility in ammonium sulfate. These differences may

---

Pepsin B 23 per cent and for egg albumin 25 per cent hydration would satisfy the requirement of spherical shape. On the basis of these considerations, these two proteins could be regarded as essentially spherical molecules, combined with not more than, respectively, 0.23 and 0.25 gm. of water per gm. of anhydrous protein.

[8] Molecular weight determinations on these globulins by ultracentrifugation and osmotic pressure methods are being carried out in cooperation with other laboratories and will be presented later.

possibly be due to differences in the degree of hydration. Further chemical investigations of these globulins will be reported later.

The molecular shape and weight of the crystalline serum albumin fractions are independent of the method of preparation for SA_A, SA_B (Kekwick), and for the most soluble crystalline fraction of the material prepared according to our modification of the Hewitt method (SA_I). For SA_α (McMeekin), however, lower values for apparent molecular asymmetry have been observed although the molecular weight is the same as that of the above fractions.

That the details of the methods of fractionation may greatly influence the purity of the final material can be seen from a comparison between the crystalline albumin fractions SA_I, SA_{II}, and

TABLE VIII

Serum Albumin SA_{II}

Solvent, 0.023 M acetate buffer, 0.2 M NaCl; pH 5.0; protein concentration 0.7 per cent.

t	D_1	D_2	D_3
sec	10^{-7}	10^{-7}	10^{-7}
15,840	5.37	5.78	6.74
28,320	5.02	5.46	6 09
45,720	4.98	5 27	6.04
80,340	5 33	5.43	6.02

SA_{III}. SA_{II} and SA_{III} differ from SA_I in that no attempt was made in the course of purification to separate the amorphous material which precipitates at pH 7 upon half saturation with ammonium sulfate. In spite of five crystallizations, these fractions were quite inhomogeneous as evidenced by the results of the diffusion measurements recorded in Table VIII for SA_{II}. Similar results have been obtained with SA_{III}. The average diffusion constants, D_1, are much too low for purified serum albumin and considerably lower than the values calculated with the method of successive analysis (D_3) in regions remote from the original diffusion boundary. Recrystallization of SA_{II} and SA_{III} at pH 5.2 slightly decreased the degree of polydispersity. The high viscosities of these preparations indicate the presence of material of higher molecular asymmetry,[9] possibly of globulin nature. Thus the

[9] The slope of the viscosity curves is primarily determined by the molecular shape distribution and is fairly independent of the particle size.

slope of the specific viscosity-volume concentration curves was 7.63 and 7.90 for SA_{II} and SA_{III} respectively, as compared with 5.71 for homogeneous material. Recrystallization of SA_{II} decreased the slope somewhat, from 7.63 to 6.45.

The seroglycoid fraction SA_D, precipitable by 2.4 to 2.5 M ammonium sulfate at pH 5.2, exhibited the same diffusion constant as the homogeneous crystalline fractions mentioned above. Its molecular asymmetry is somewhat higher, and hence the calculated molecular weight somewhat lower, in agreement with the observations of Rimington and Van den Ende (43).

SUMMARY

By combining the methods of viscosity and diffusion it is possible to estimate the shape and weight of anhydrous protein molecules, provided the limitations considered in the theoretical part of this paper are observed.

The relative viscosities of solutions of a number of proteins have been found to be independent of the velocity gradient of flow, and to be linear with respect to the protein concentration up to about 11 mg. per cc. From the slope of the curves obtained when the specific viscosity is plotted against the volume concentration, the asymmetries of the proteins, hydration being neglected, have been calculated with the Polson and Simha equations. Comparison of the dissymmetry constants and molecular weights as obtained from sedimentation and diffusion measurements with those calculated with the Perrin equation from viscosity and diffusion data indicates the applicability of Simha's equation when solved for the model of prolate ellipsoids. The assumption of proteins resembling in shape oblate ellipsoids gives rise to dissymmetry constants considerably higher, and molecular weights considerably lower than those observed. Polson's empirical viscosity equation has been shown in this paper not to be strictly applicable if viscosities are measured in dilute protein solutions.

The influence of hydration on apparent molecular asymmetry

has been considered, and probable values for true asymmetries, assuming about 33 per cent hydration, are given.

Quantitative viscosity and diffusion measurements are reported for crystalline lactoglobulin, two fractions of crystalline pepsin, a number of crystalline and one amorphous fraction of serum albumin, and three fractions of water-soluble horse pseudoglobulin.

A method is described for isolating apparently monodisperse preparations of normal euglobulin-free pseudoglobulin. They have been defined chemically by nitrogen and carbohydrate content, and physically by molecular weight and shape.

Comparative observations on crystalline serum albumin prepared according to the methods of Kekwick, McMeekin, and a new modification of that by Hewitt have been carried out.

An apparently monodisperse seroglycoid fraction of low carbohydrate content has been isolated and also subjected to chemical and physical measurements.

BIBLIOGRAPHY

1. Neurath, H., *J. Am. Chem. Soc.*, **61**, 1841 (1939).
2. Neurath, H , and Cooper, G. R , *J. Am. Chem. Soc.*, **62**, 2248 (1940).
3. Einstein, A , *Ann. Physik*, **19**, 289 (1906); **34**, 591 (1911).
4. Perrin, F., *J. physiq. radium*, 7, 1 (1936).
5. Herzog, R. O., Illig, R., and Kudar, H., *Z. physik. Chem.*, *Abt. A*, **167**, 329 (1934)
6. Neurath, H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 196 (1938).
7. Polson, A., *Nature*, **137**, 740 (1936); *Kolloid-Z.*, **88**, 51 (1939).
8. Jeffery, G. B., *Proc. Roy. Soc. London, Series A*, **102**, 163 (1923).
9. Simha, R., *J. Physic. Chem.*, **44**, 25 (1940).
10. Adair, G. S., and Adair, M. E., *Proc. Roy. Soc. London, Series B*, **120**, 422 (1936); *Tr. Faraday Soc.*, **36**, 23 (1940).
11. Kraemer, E. O., *J. Franklin Inst.*, **229**, 393, 680 (1940).
12. For references see Greenberg, D. M., in Schmidt, C. L. A., The chemistry of the amino acids and proteins, Springfield, 451 (1938).
13. Daniel, J., and Cohn, E. J., *J. Am. Chem. Soc.*, **58**, 415 (1936).
14. Fahey, K. R., and Green, A. A., *J. Am. Chem. Soc.*, **60**, 3039 (1938).
15. Neurath, H., and Saum, A. M., *J. Biol. Chem.*, **128**, 347 (1939).
16. Bull, H. B., *J. Biol. Chem.*, **133**, 39 (1940).
17. Polson, A., *Kolloid-Z.*, **87**, 149 (1939).
18. Pedersen, K. O., in Svedberg, T., and Pedersen, K. O., The ultracentrifuge, New York, 354 ff. (1940).
19. Burgers, J. M., in Second report on viscosity and plasticity, New York, 113 ff. (1938).

20. Guth, E., *Kolloid-Z.*, **74**, 147 (1936); **75**, 15 (1936).   Guth, E., and Simha, R., *Kolloid-Z.*, **74**, 266 (1936).   Eirich, F., and Simha, R., *Monatsh. Chem.*, **71**, 67 (1937).   Simha, R., *Kolloid-Z.*, **76**, 16 (1936).

21. Huggins, M. L., *J. Physic. Chem.*, **42**, 911 (1938); **43**, 439 (1939).

22. Peterlin, A., *Z. Physik*, **111**, 232 (1938); *Kolloid-Z.*, **86**, 230 (1939). Peterlin, A., and Stuart, H. A., *Z. Physik*, **112**, 129 (1939).

23. Robinson, J R., *Proc. Roy. Soc. London, Series A*, **170**, 519 (1939).

24. Bungenberg de Jong, H. G., in First report on viscosity and plasticity, New York, 131 (1935).

25. Philippoff, W., *Kolloid-Z.*, **75**, 142, 155 (1936).

26. Eirich, F., and Goldschmid, O., *Kolloid-Z.*, **81**, 7 (1937)

27. For references see Bredée, H. L., and de Booys, J., *Kolloid-Z* , **79**, 31, 43 (1937).

28. Kunitz, M., *J. Gen. Physiol.*, **9**, 715 (1926); **10**, 811 (1926–27).

29. Treffers, H. P., *J. Am. Chem. Soc.*, **62**, 1405 (1940).

30. Edsall, J. T., and Mehl, J. W., *J. Biol. Chem.*, **133**, 409 (1940).

31. Kuhn, W., *Z. physik. Chem.*, *Abt. A*, **161**, 1 (1932).

32. Mehl, J. W., Oncley, J. L., and Simha, R., *Science*, **92**, 132 (1940).

33. Jones, G., and Fornwalt, H. J., *J. Am. Chem. Soc.*, **60**, 1683 (1938).

34. Lamm, O., *Nova acta regiae soc. scient. Upsaliensis*, **10**, No. 6 (1937).

35. Neurath, H., and Cooper, G. R., *J. Biol. Chem.*, **135**, 455 (1940).

36. Philpot, J. St. L., *Biochem. J.*, **29**, 2458 (1935)

37. Steinhardt, J., *J. Biol. Chem.*, **129**, 135 (1939).

38. Green, A. A., *J. Am. Chem. Soc.*, **60**, 1108 (1938).

39. McMeekin, T. L., *J. Am. Chem. Soc* , **61**, 2884 (1939).

40. Sørensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933); *Compt.-rend. trav. Lab. Carlsberg*, **19**, No. 12 (1933).

41. Hewitt, L. F., *Biochem. J.*, **30**, 2229 (1936); **31**, 360, 1534 (1937); **32**, 26 (1938); **33**, 1496 (1939).

42. Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938).

43. Rimington, C., and Van den Ende, M., *Biochem. J.*, **34**, 941 (1940).

44. Kraemer, E. O., in Svedberg, T., and Pedersen, K. O., The ultracentrifuge, New York, 65 (1940).

45. Kraemer, E. O., and Sears, G. R., *J. Rheol.*, **1**, 231 (1930).

46. Neurath, H., and Bull, H. B., *J. Biol. Chem.*, **115**, 519 (1936).

47. Hatschek, E., *Tr. Faraday Soc.*, **32**, 787 (1936).

48. Tiselius, A., *Biochem. J.*, **31**, 1464 (1937).

49. Pappenheimer, A. M., Lundgren, H. P., and Williams, J. W., *J. Exp. Med.*, **71**, 247 (1940).

LETTERS TO THE EDITORS

BIOLOGICAL RELATIONSHIPS OF CHOLINE, ETHANOL-AMINE, AND RELATED COMPOUNDS*

Sirs:

The biological relationships of the phosphatide bases ethanolamine and choline with each other and with glycine and betaine have been investigated with the aid of N^{15}. Isotopic ethanolamine, choline and glycine,[1] and betaine, prepared by the methylation[2] of isotopic glycine, were fed at levels corresponding to 21 mg. of N per rat per day for 3 days to adult (250 gm.) rats on a standard basal diet containing 15 per cent casein. The rats were then killed; pure ethanolamine and choline, obtained from the total crude phosphatides by a modification of the method of Thierfelder and Schultze,[3] glycine and glutamic acid from the proteins of the combined internal organs, and urea and ammonia from the urine were analyzed for N^{15}.

In all cases the urinary ammonia and urea contained appreciable amounts of isotope, indicating that each test substance was partially degraded by the animal. Another portion of the isotope was introduced into the phosphatides. Dietary ethanolamine and choline were found to have replaced 28 per cent and 21 per cent respectively of these components of the total phosphatide. The phosphatide choline isolated after ethanolamine feeding was very rich, whereas the phosphatide ethanolamine isolated after choline feeding was very poor in isotope. Glycine isolated from the organ proteins after feeding ethanolamine contained only traces of marked nitrogen, but, when isotopic glycine was fed, the resulting phosphatide ethanolamine contained much N^{15}.

When isotopic betaine was fed, the glycine of the organ proteins

[1] Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939). Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **138**, 167 (1941).

[2] Novak, J., *Ber. chem. Ges.*, **45**, 834 (1912).

[3] Thierfelder, H., and Schultze, O., *Z. physiol. Chem.*, **96**, 296 (1915).

and the phosphatide ethanolamine were almost as rich in N^{15} as the corresponding fractions of the glycine feeding. On the other hand, the phosphatide choline contained no more isotope than could well be accounted for by the conversion *in vivo* of ethanolamine to choline, indicated above. It thus appears that dietary betaine, like sarcosine,[4] is rapidly demethylated to glycine, little or none of it being directly reduced to choline.

The results of these observations may be formulated as follows:

Betaine ⟶ glycine ⟶ ethanolamine ⟶ choline
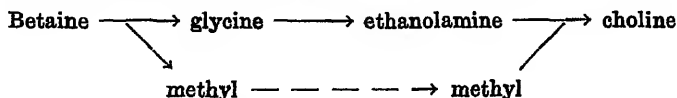
methyl — — — — ⟶ methyl

None of these steps was found to proceed in the reverse direction. The chief source of the methyl groups in the last step, when the animals are kept on a normal diet, is doubtless methionine, as shown by du Vigneaud *et al.*[5] The lipotropic activity of betaine is mainly attributable to its function as a donor of methyl groups in choline synthesis. This likelihood is indicated in the above scheme by a broken line.

Department of Biochemistry   DeWitt Stetten, Jr.
  College of Physicians and Surgeons
  Columbia University
  New York

---

[4] Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 99 (1940).

[5] du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).

DETERMINATION OF THE BASES OF PHOSPHOLIPIDS BY THE ISOTOPE DILUTION METHOD*

Sirs:

The interesting report[1] of the presence of a hydroxyamino acid in brain cephalin makes it advisable to present results obtained in this laboratory. In the course of work on the rate of phosphatide synthesis in various tissues[2] and on the blood-clotting activity of different tissue phosphatides it became increasingly evident that the conception of the structure of cephalins as aminoethyl phosphoryl glycerides (differing only in the types of fatty acids present) did not fit the actual situation.

As a preliminary step to the search in phosphatides for bases other than ethanolamine and choline the distribution of these known constituents in organ phosphatides was determined by the isotope dilution method.[3] The pure bases containing a known amount of the N^{15} isotope (2.00 atom per cent N^{15} excess) were added to the phosphatide hydrolysis mixture. The isotope dilution found in pure specimens of the compounds subsequently isolated from the hydrolysates permits an accurate estimation of the amounts originally present in the phosphatides, provided complete hydrolysis has taken place. The alcohol-insoluble barium salts isolated from the hydrolysate were practically free of nitrogen.

The phospholipids examined were obtained from *pig heart* and *pig liver*. After the customary purification the phosphatides used gave satisfactory analyses and had a N:P ratio of 1:1 (see the table).

For the isolation of the bases, derivatives were selected which

[1] Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).

[2] Chargaff, E., *J. Biol. Chem.*, **128**, 587 (1939). Chargaff, E., Olson, K. B., and Partington, P. F., *J. Biol. Chem.*, **134**, 505 (1940).

[3] Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).

contained no other nitrogen than that of the base and could easily be purified.   The purity of the compounds was checked by chemical analysis and melting point determination.   Ethanolamine was isolated as diiodosalicylate,[4] which proved a useful derivative for this purpose; choline was isolated as the $6HgCl_2$ double salt.

| Phosphatide | P | N | NH_2-N | N:P | Phosphatide used for hydrolysis | Ethanolamine | | Choline | | Ethanolamine N in per cent of amino N | Choline N in per cent of non-amino N |
						Added*	N^{15} excess in compound isolated	Added*	N^{15} excess in compound isolated		
	per cent	per cent	per cent		gm.	mg.	atom per cent	mg.	atom per cent		
Pig heart	3.70	1 58	1.17	1:1.05	5.0317	56.6	0.598	23.5	0.257	51.5	92 3
" liver	3.70	1 65	1.22	1:1.01	5.0900	80.9	0.882			37 9	

* Both bases added contained 2.00 atom per cent N^{15} excess.

The results are summarized in the accompanying table.   The experiments, which are being continued, show that, *whereas choline accounts for practically all the non-amino nitrogen in the phosphatide hydrolysates, only 40 to 50 per cent of the amino nitrogen is present as ethanolamine and that the occurrence of at least one other primary base or hydroxyamino acid must be assumed.*

Departments of Biochemistry and Surgery   ERWIN CHARGAFF
    College of Physicians and Surgeons   MORRIS ZIFF
    Columbia University   D. RITTENBERG
    New York

[4] Chargaff, E., unpublished results.

CONFIRMATORY EVIDENCE OF THE CHROMOTRICH-
IAL ACTIVITY OF p-AMINOBENZOIC ACID

Sirs:

In a paper on hydroquinone poisoning, Oettel[1] reported that cats gradually develop graying of the fur when receiving hydroquinone. This observation was confirmed by one of us (G. J. M.) and experiments started with young mice weighing 10 to 12 gm. The animals were placed on the McCollum stock diet[2] to which hydroquinone had been added at the rate of 100 mg. per kilo of diet. As previously reported[3] most of the animals showed definite achromotrichia within a period of from 4 to 20 weeks, and cure could be brought about by feeding a rice polish concentrate as a daily supplement.

The curative effect of p-aminobenzoic acid was investigated at the suggestion of one of us (S. A.). We administered 0.25 ml. of Preparation x-1, containing 3 mg. per ml. of p-aminobenzoic acid, as recently described,[4] daily to thirty mice with typical achromotrichia. After about a week some of the animals showed definite signs of blackening of the fur, and at the end of 2 weeks all the mice were cured. In fact, their fur appeared to be more deeply pigmented than that of the animals on stock diet.

The experiments as a whole seem to justify the previous conclusions that p-aminobenzoic acid is a chromotrichia factor,[4] and that hydroquinone achromotrichia is a vitamin deficiency.[3] Administered at a given level, p-aminobenzoic acid appears to have a more rapid and more pronounced effect than the rice polish concentrate.

Warner Institute for Therapeutic Research   Gustav J. Martin
    New York
The Squibb Institute for Medical Research   S. Ansbacher
    New Brunswick

[1] Oettel, H., *Arch. exp. Path. u. Pharmakol.*, **183**, 319 (1936).

[2] Wheat, maize, rolled oats, flaxseed oil meal, casein, whole milk powder, calcium carbonate, sodium chloride, iron citrate, and copper sulfate.

[3] Martin, G. J., Proceedings of the American Association for the Advancement of Science, Pharmacy Section, January (1941).

[4] Ansbacher, S., *Science*, **93**, 164 (1941).

ELIMINATION OF ACETOACETIC ACID IN THE DETERMINATION OF PYRUVIC ACID BY LU'S METHOD

Sirs:

The method of Lu[1] for the determination of pyruvic acid is limited in its specificity because other keto acids yield the same color as pyruvic acid. Thus, in the presence of significant amounts of acetoacetic acid, false values may be obtained unless some means is taken to remove the acetoacetic acid.

The removal of acetoacetic acid from blood containing pyruvic acid is made possible by the following procedure. 0.1 to 0.5 ml. of blood, stabilized with sodium iodoacetate, is precipitated with 4.5 ml. of 0.0747 N H_2SO_4 and 1.0 ml. of 5 per cent sodium tungstate. After centrifugation and quantitative removal of the supernatant, the precipitate is washed with 2 ml. of a solution

Treatment	Initial pyruvic acid	Acetoacetate added	Final pyruvic acid
	mg. per cent	*mg. per cent**	*mg. per cent*
Standard solutions			
HCl.....	2.48	0	2.47
" heating......	2.48	0	2.47
None.................	2.48	2.76	5.24
HCl.....	2.48	2.76	4.30
" heating.......	2.48	2.76	2.46
Blood			
HCl, heating	2.35	21.8	2.40
" "	3.79	21.8	3.79

* As pyruvic acid; this value × 49 yields mg. per cent of acetoacetic acid.

consisting of 4.5 ml. of 0.0747 N H_2SO_4, 1.0 ml. of sodium tungstate, and 0.5 ml. of H_2O. To the combined supernatants in a 15 ml. centrifuge tube 0.50 ml. of concentrated HCl (sp. gr. 1.1878) is added and the tube placed in a boiling water bath for

[1] Lu, G. D., *Biochem J.*, **33**, 249 (1939).

1 hour.   An amount of 40 per cent NaOH which will just neutral-
ize the HCl is added.   The solution is allowed to cool; 2,4-dini-
trophenylhydrazine solution is added and the method of Lu is
followed thereafter.

Results obtained with this procedure are illustrated in the table
and indicate the efficiency of this step.

The above procedure is applicable to other biological materials.

May Institute for Medical Research, the Jewish   SAMUEL ELGART
   Hospital, and the Department of Biochemistry,   NORTON NELSON
   University of Cincinnati
   Cincinnati

SULFUR IN PROTEINS

VI. QUALITATIVE STUDIES IN THE ALKALINE DECOMPOSITION OF CYSTINE*

By HAROLD V. LINDSTROM and W. M. SANDSTROM

(*From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul*)

Quantitatively it has been shown that, when cystine is boiled in alkaline solution, desulfurization to H_2S and deamination to NH_3 are never complete. The alkaline earth hydroxides were found to be more destructive than the alkali hydroxides.

Qualitatively it has been shown that cystine in boiling alkali will give H_2S, S, $H_2S_2O_3$, H_2SO_3, CO_2, NH_3, oxalic acid, and non-volatile liquid acids; a product resembling uvitic acid was isolated by Baumann (1); and *dl*-alanine was isolated by Mauthner (2) from a lot in which cystine had been kept for 2 months in cold NH_4OH containing zinc dust. Gortner and Sinclair (3) boiled cystine in $Ba(OH)_2$ for 24 hours and were able to isolate only hygroscopic material from which it was difficult to identify organic products. Clarke and Inouye (4, 5) demonstrated that pyruvic acid was a primary decomposition product of cystine and showed that this pyruvic acid accounted for the rapid destruction of cystine in the manner of an autocatalytic reaction. In spite of the destructive nature of the pyruvic acid generated, some decomposition product of cystine stabilized residual cystine, as shown by the data of Thor and Gortner (6). Herbst (7) boiled ethyl cysteine with pyruvic acid in water solution to obtain alanine, CO_2, CH_3CHO, and ethyl thioglycolic aldehyde.

The purpose of the present investigation was to identify the organic products of decomposition and to explain why cystine is stabilized by its own decomposition products.

50 gm. samples of tyrosine-free cystine were boiled under a reflux with 1 liter of 2 N $Ba(OH)_2$ for 24 hours. The cooled solution was filtered and suction was applied to the filtrate to remove as much NH_3 as possible, after which barium was removed with H_2SO_4. The barium-free and sulfate-free solution was concentrated *in vacuo* to a hygroscopic syrup which was found to be acidic, to contain non-cysteine —SH groups, and to have strong reducing powers. When neutralized with $BaCO_3$, there was obtained an alcohol-insoluble but water-soluble barium compound which was dry and stable in air. A 25 gm. portion of this powder was treated with concentrated HNO_3 and the mixture was evaporated on the steam bath to a pale yellow syrup together with a white solid mass. Extraction with ether removed two solid acids, oxalic acid and water-insoluble uvitic acid (m.p. 290°, methyl ester m.p. 97°). The ether-insoluble residue was extracted with boiling water to remove uvitonic acid (2-methyl-4,6-dicarboxy-pyridine), insoluble in cold water, and an acid syrup containing sulfonic acid groups, which was soluble in cold water. The uvitonic acid was identified by its melting point (274°) and by the fact that on heating with CaO it gave α-picoline (identified as the picrate, m.p. 162°). The ether-insoluble and hot water-insoluble mass proved to be barium sulfate. Since the nitric acid treatment destroyed most of the organic sulfur acids in the syrup, the untreated syrup was investigated. Ether removed an acid syrup rich in sulfhydryl groups but identification was difficult because of the poor yield and impurity of extract.

In a control experiment, in place of cystine the three primary decomposition products (pyruvic acid, H_2S, NH_3) were added to boiling barium hydroxide at a rate approximating their rate of generation in cystine decomposition. After all had been added, the boiling was continued for 5 hours to make sure that only those products stable to boiling alkali would be obtained in the hygroscopic residue. By this means enough ether-soluble syrup was obtained for identification; this syrup was dissolved in water,

reduced with tin and hydrochloric acid, and identified as thiolactic acid by use of the benzyl derivative (m.p. 73°) in the manner of Suter (8). Uvitic and uvitonic acids were also found, as well as thiolactic acid.

In attempts to isolate alanine from the decomposition products of cystine, the nitric acid treatment could not be used. The organic sulfur was partially removed by ether extraction and then by exhaustively boiling with alkaline lead acetate in which alanine is stable. The excess lead was removed with hydrogen sulfide; the filtrate was neutralized with sodium carbonate, concentrated, and treated with alcohol. Analysis of the resulting precipitate indicated total nitrogen equivalent to 26.7 per cent and amino

TABLE I

Sullivan Color Values Showing Stabilization of Cystine by Alanine

Molar ratio of alanine to cystine	Ratio of color value to that with cystine alone
0	1.00
1	1.03
2	1.03
5	1.19
10	1.32
15	1.06
20	0.86

nitrogen equivalent to 28.9 per cent of alanine. Estimation of alanine by the method of Fürth (9) showed 27.5 per cent. The presence of glycine, shown by Daft and Coghill (10) to be produced in the alkaline decomposition of serine, thus appears unlikely. The alcoholic filtrate on treatment with acetone yielded a sticky precipitate which also was rich in alanine, but attempts to prepare a crystalline benzoyl derivative from it were unsuccessful.

These results made it appear possible that cystine is stabilized in boiling alkaline solutions not by the formation of a "cystine-X" complex (6) but by the inactivation of pyruvic acid by its conversion into a Schiff base with alanine. To test this hypothesis, a series of experiments was conducted in which cystine was heated under standard conditions with alkali alone and in the presence

of alanine.   Mixtures of 0.100 gm. of cystine with various amounts
of the sodium salt of alanine were made up to 25 cc. with 2 N
sodium hydroxide.   These solutions were heated on the steam
bath for 2 hours, as in the experiments of Thor and Gortner (6).
The relative amounts of unchanged cystine (Table I) were de-
termined by the colorimetric method of Sullivan (11), which is
the only available procedure applicable in the presence of sodium
sulfide and sulfhydryl compounds (such as thiolactic acid).   In
control experiments in which the heating was omitted no change
in color intensity was induced by the addition of alanine up to 10 M
proportions, but with 15 M and more a progressive decrease was
found.   This explains the low ratios observed when cystine was
heated in alkaline solution with 15 and 20 moles of alanine.

Similar results were obtained in comparable experiments with
potassium hydroxide, but with barium hydroxide no increase
in the color ratio with increasing amounts of alanine was observed.

<div align="center">DISCUSSION</div>

Cystine breakdown can be pictured as taking place in two main
steps.   The first is the removal of sulfur as H_2S and nitrogen as
NH_3 to leave pyruvic acid, and is a relatively slow reaction.   The
pyruvic acid is subject to several competing reactions; one of these
is the combination of the carbonyl group of the pyruvic acid with
the amino group of cystine to split out water and leave a Schiff
base type (I).   The formation and resultant breakdown of the

$$HOOC-CH-CH_2-SH \;+\quad\quad\quad O$$
$$\quad\quad\quad\; | \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad ||$$
$$\quad\quad\quad NH_2 \quad\quad\quad\quad HOOC-C-CH_3 \quad\longrightarrow$$

$$HOOC-CH-CH_2-SH \;+\; H_2O$$
$$\quad\quad\quad\quad | $$
$$\quad\quad\quad\quad N$$
$$\quad\quad\quad\quad ||$$
$$\quad\quad HOOC-C-CH_3$$

<div align="center">(I)</div>
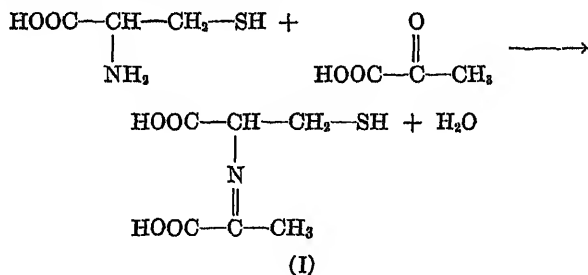
Schiff base form the second step and are relatively fast reactions.
Since the breakdown results, as shown by the mechanism of
Herbst (7), in the formation of aldehydes (acetaldehyde and
thioglycolic aldehyde) which themselves promote the decomposi-

tion of cystine, the resulting increase in the rate of cystine decomposition follows the curve of an autocatalytic reaction (II) as shown by Clarke and Inouye (4). Alanine is formed from the

$$HOOC\text{—}CH\text{—}CH_2\text{—}SH$$
$$|$$
$$N$$
$$\|$$
$$HOOC\text{—}C\text{—}CH_3$$

$$\underset{\longleftarrow}{\longrightarrow}$$

$$HOOC\text{—}C\text{—}CH_2\text{—}SH$$
$$\|$$
$$N$$
$$|$$
$$HOOC\text{—}CH\text{—}CH_3$$

$$\downarrow H_2O$$

$$\downarrow H_2O$$

$$CO_2 + CH_3CHO +$$
$$HOOC\text{—}CH(NH_2)\text{—}CH_2\text{—}SH$$

$$CO_2 + HS\text{—}CH_2\text{—}CHO +$$
$$CH_3\text{—}CH(NH_2)\text{—}COOH$$

(II)
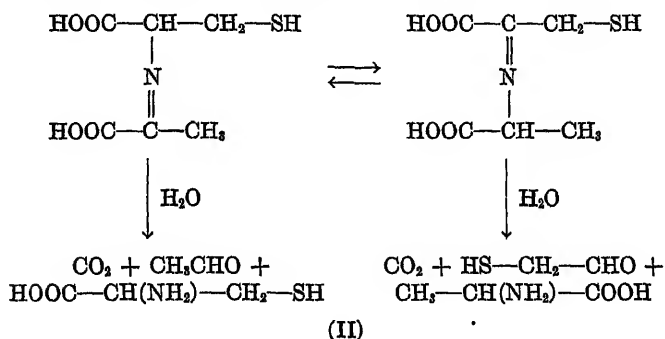
Schiff base breakdown and it can combine with pyruvic acid in the same manner as does cystine, but the net result in this case is the splitting of pyruvic acid to carbon dioxide and acetaldehyde

$$CH_3\text{—}CH(NH_2)\text{—}COOH + CH_3\text{—}CO\text{—}COOH \xrightarrow{H_2O}$$

$$HOOC\text{—}CH\text{—}CH_3$$
$$|$$
$$N$$
$$\|$$
$$HOOC\text{—}C\text{—}CH_3 \qquad H_2O$$
$$\updownarrow \qquad\qquad\qquad \longrightarrow \quad CH_3\text{—}CH(NH_2)\text{—}COOH +$$
$$HOOC\text{—}C\text{—}CH_3 \qquad\qquad\qquad CO_2 + CH_3CHO$$
$$\|$$
$$N \qquad\qquad H_2O$$
$$|$$
$$HOOC\text{—}CH\text{—}CH_3$$

(III)
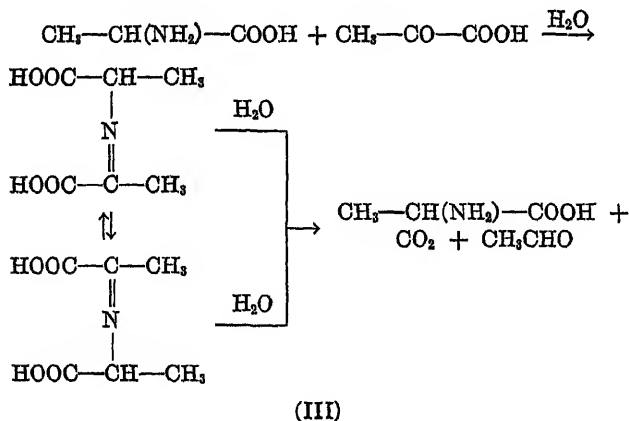
(III). The alanine is not destroyed but accumulates as cystine disappears, and with increasing effectiveness competes with the cystine for the pyruvic acid, at which time the decomposition of cystine due to Schiff base formation becomes very small.

The apparent paradox, that the generally more active alkali metal hydroxides decompose cystine at a slower rate than barium hydroxide (6), can be resolved by taking into consideration their greater activity in bringing about the condensation of destructive

carbonyl groups.   The decrease in the rate of cystine decomposition as the concentration of sodium hydroxide or potassium hydroxide increases (6) can be similarly explained.

SUMMARY

Uvitic, uvitonic, and thiolactic acids were identified for the first time as alkaline decomposition products of cystine and it was shown that they arose from the action of the alkali on the primary decomposition products, pyruvic acid, NH_3, and H_2S.

Alanine was demonstrated as a decomposition product by quantitative analyses on a powder isolated from a barium hydroxide decomposition.   Since alanine was found to stabilize cystine somewhat in sodium hydroxide and potassium hydroxide but not in barium hydroxide solutions, a theory was put forth that alanine stabilized cystine in alkaline solutions, the alanine arising from the decomposition of cystine and inhibiting the action of the cystine-labilizing factor, pyruvic acid.   The result is that alanine is resynthesized and pyruvic acid (as well as any acetaldehyde derived from it) is removed by being condensed rapidly in sodium hydroxide or potassium hydroxide but only slowly in barium hydroxide.

BIBLIOGRAPHY

1. Baumann, E., *Ber. chem. Ges* , **15**, 1731 (1882).
2. Mauthner, J., *Z. physiol. Chem.*, **78**, 28 (1912).
3. Gortner, R. A., and Sinclair, W. B., *J. Biol. Chem.*, **83**, 681 (1929).
4. Clarke, H. T., and Inouye, J. M., *J. Biol. Chem.*, **89**, 399 (1930).
5. Clarke, H. T., and Inouye, J. M., *J. Biol. Chem* , **94**, 541 (1931-32).
6. Thor, C. J. B., and Gortner, R. A., *J. Biol. Chem.*, **99**, 383 (1932-33).
7. Herbst, R. M., *J. Am. Chem. Soc.*, **58**, 2239 (1936).
8. Suter, F., *Z. physiol. Chem.*, **20**, 564 (1895).
9. Fürth, O., Scholl, R., and Herrmann, H., *Biochem. Z.*, **251**, 404 (1932).
10. Daft, F. S., and Coghill, R. D., *J. Biol. Chem.*, **90**, 341 (1931).
11. Sullivan, M. X., *Pub. Health Rep.*, *U. S. P. H. S.*, **44**, 1421 (1929).

THE ADSORPTION OF PHOSPHATES AT FORTY DEGREES BY ENAMEL, DENTIN, BONE, AND HYDROXYAPATITE AS SHOWN BY THE RADIOACTIVE ISOTOPE*

By HAROLD CARPENTER HODGE, GRANT VAN HUYSEN, JOHN F. BONNER, AND STANLEY N. VAN VOORHIS

(*From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, and the Department of Physics, College of Arts and Science, The University of Rochester, Rochester, New York*)

Adsorption of phosphates by calcified tissues may occur from the potassium hydroxide-ethylene glycol solutions used to extract the organic portion of tooth and bone substance (1, 2). Since it has recently been shown that dentin and enamel exchange phosphates with aqueous phosphate solutions (3), it is of interest to determine whether these systems are described by adsorption isotherms.

Procedure

Radioactive phosphate solutions were prepared from bombarded red phosphorus as has been described previously (1). Aliquots from these solutions were suitably diluted; the activities determined by the Geiger-Müller scale-of-four counter (4) were used as standards for calculating the experimental values.

The samples used were powdered (60 mesh) bone, dentin, and enamel. The bone and enamel samples had been glycol-ashed (2); the dentin was the unashed product of the centrifugal flotation separation process (5). The hydroxyapatite was Sample H1 (Ca:P ratio 2.10) described by Hodge, LeFevre, and Bale (6). It was not screened.

Suitable aliquots of the radioactive phosphate solution were made up to 50 cc. with solutions of disodium acid phosphate

which varied in concentration from 0.2 to 0.00002 M by powers of 10. 25 cc. of the phosphate solutions containing radioactive phosphorus were stirred with 50 mg. samples of bone, dentin, enamel, or hydroxyapatite for 30 minutes at 40°. The solutions were decanted after centrifugation and the precipitates washed twice with distilled water. The precipitates were dissolved in about 3 cc. of normal hydrochloric acid. The counting procedure has been described previously (7) as well as the methods of calculation.

Data

In Table I are given the mg. of phosphorus picked up per gm. of bone, hydroxyapatite, dentin, and enamel at each of the final concentrations of phosphate found. There is a regular progression

TABLE I

Phosphorus Picked Up by Various Calcium Phosphates from Solutions of Sodium Phosphate (0.2 to 0.00002 M)

Equilibrium, M concentration of phosphate (approximate)	Average P picked up per gm. solid				Log X/M			
	Bone	Apatite	Dentin	Enamel	Bone	Apatite	Dentin	Enamel
	mg.	*mg.*	*mg.*	*mg.*				
1.6×10^{-1}	55.2	45.7	26 1	19.8	1.74	1.66	1.42	1.29
1.6×10^{-2}	24.1	19.1	14 1	5 4	1 38	1.28	1.15	0.73
1.6×10^{-3}	4.4	1.8	0.9	1.3	0.64	0.25	−0.03	0.11
1.5×10^{-4}	1.2	0.7	0 3	0 1	0.06	−0.16	−0.52	−0.89
1.4×10^{-5}	0.2	0.1	0.07	0.04	−0 71	−0 85	−1.15	−1.43

for each substance; 20 to 50 mg. of phosphorus are picked up per gm. of solid exposed to the highest phosphate concentration and 0.04 to 0.2 mg. is picked up from the most dilute phosphate solution. Expressed as percentages of the total phosphate in the solution initially, the solids picked up approximately 1, 1, 4, 10, and 20 per cent from the solutions in order of increasing dilution.

When the logarithms of the mg. of phosphorus picked up per gm. of sample ($\log_{10} X/M$) are plotted as ordinates against the logarithm of the molar concentration of phosphate remaining in the solution at equilibrium ($\log_{10} C$), a straight line is obtained (Fig. 1) for each solid. These lines are not parallel; instead they diverge in the lower concentration ranges. However, since a

linear function is obtained for each calcium phosphate, this criterion of adsorption is satisfied and each substance may be said to adsorb phosphates under the conditions of the experiment. The equation for each of these lines was calculated; the n and k
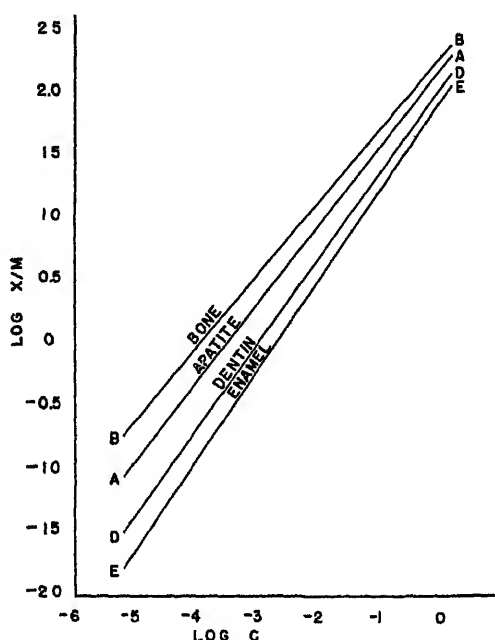
Fig. 1. Adsorption isotherms at 40° for phosphates on various calcium phosphates.

values for the Freundlich adsorption equation $\log_{10} X/M = \log_{10} k + 1/n \log_{10} C$ are as follows:

	Bone	Apatite	Dentin	Enamel
n	1.71	1.59	1.47	1.40
k	209	166	115	87

DISCUSSION

The isotherms (Fig. 1) show that bone, dentin, and enamel adsorb phosphates from aqueous solutions at approximately body temperature. Furthermore, these calcified tissues adsorb phos-

phates in a fashion mathematically comparable to the adsorption by a synthetic hydroxyapatite. This evidence supports the concept (6) that the principal inorganic molecule of tooth and bone substance is hydroxyapatite upon the minute crystal surfaces of which sufficient phosphates are adsorbed to account (at least in part) for the calcium to phosphorus ratio of 2.10 (theoretical Ca:P ratio for hydroxyapatite, 2.15).

In Fig. 1, the location of the isotherms indicates that in order of their ability to adsorb phosphates bone > dentin > enamel. Armstrong (3) has recently compared the ability of dentin and enamel to pick up phosphate from aqueous solutions at 37°. His data for dentin (log X/M = 1.1, log C = -1.2) fall almost exactly on our isotherm for dentin; however, his data for enamel (log X/M = $+0.2$, log C = -1.2) fall below our isotherm. Manly and Levy (1) have reported that, from ethylene glycol solution at 200° under conditions like those used in ashing calcified tissues, the calcified tissues adsorb phosphates in the order bone > dentin > enamel. Volker *et al.* (7) have reported the same order in the ability of these tissues to adsorb fluorides. The k value, characteristic of the adsorbent, expresses the adsorbing power quantitatively. When the k for enamel is set at unity, the k values for bone and dentin are as follows:

Adsorption conditions	Bone	Dentin
Phosphate at 40°.	2.4	1.3
" " 200° (1)	6.3	5.2
Fluoride " 40° (6).	2.3	1.9

Undoubtedly this order arises from some fundamental difference in the crystals. One known physical difference is in the size of the ultimate crystals; these are much smaller in dentin and bone (10^{-5} to 10^{-6} cm.) than in enamel (10^{-4} cm.). The smaller crystals would have a larger effective adsorbing surface and should show a greater adsorbing power (k). The greater density of enamel (2.9) as compared to dentin (2.2) or bone (about 2.0) would limit the permeability of this tissue to aqueous solutions and thus tend also to give a lower rate of phosphate exchange.

When the data on phosphate adsorption at 40° are compared with those at 200°, two facts are obtained. First, there is (as

would be expected) less phosphate adsorbed at 200° than at 40°; e.g., for bone at 200°, k is 8.8; at 40°, k is 209. Second, the isotherms at 200° are approximately parallel to those at 40°; e.g., for bone at 200°, n is 1.6; at 40°, n is 1.7.

The delicateness of this radiophosphate method should be emphasized. The data for 50 mg. samples of bone in a 0.00002 M phosphate solution showed from eight analyses that an average of 9.6 γ of phosphorus were picked up. The eight analyses varied from this average by only 1 per cent (average deviation). To have established this fact chemically would have called for the difficult task of distinguishing between 4.25 and 4.26 mg. of phosphorus in each 50 mg. bone sample before and after exposure to the phosphate solution.

The concept of a mineral exchange in bone has been widely accepted. In bone (and probably in dentin) part of the phosphates is assumed to be in equilibrium with the blood phosphates (8). As the blood radiophosphorus level rises and falls, the labile bone P^{32} level also rises and falls. This rapid turnover may involve several equilibria in which adsorption plays a part. Exchange adsorption would increase the bone radiophosphorus level by exchanging P^{31} atoms of the surfaces of the minute crystals with P^{32} atoms of the blood. This process probably accounts in part for the rapid pick-up of P^{32} by various calcified tissues (9). Part of the normal mineral metabolism of labile bone may be a solution and reprecipitation of calcium phosphates. In these reactions, radiophosphorus would fit into the hydroxyapatite lattice in the place of P^{31} atoms. Furthermore, the new crystals would present surfaces upon which P^{32} atoms could be adsorbed in the ratio $P^{32}:P^{31}$ currently in the blood.

In bone, the adsorption processes would be of some importance owing to the large fraction shown to be labile (one-sixth of epiphyseal bone in young rats) (8). However, in enamel, the slow interchange of fluids (by diffusion) would permit contact of radiophosphorus with a much smaller surface in a given time. Thus, a negligible turnover of radiophosphorus would be expected and has been described (10). On the other hand, if phosphate solutions (e.g. the saliva) with high $P^{32}:P^{31}$ ratios were placed in contact with the teeth, even the relatively small surface exposed should adsorb sufficient P^{32} to give a discernible radioactivity. Volker

and Sognnaes (11) have observed higher P^{32} values for surface than for inner layers of enamel and suggest that adsorption is a factor. Calculations with the data of Table I have shown that the salivary levels ($P^{32}:P^{31}$) may be sufficient to account for as much P^{32} as has been found in surface enamel specimens. In these calculations it is assumed that area for area the phosphate is adsorbed in the proportions observed for powdered enamel samples. Thus, adsorption of phosphates may be in part the explanation for the finding of von Hevesy and Armstrong (12) that there are small but detectable activities in the enamel of the teeth of cats following the subcutaneous administration of highly radioactive phosphate solutions.

SUMMARY

1. Bone, dentin, and enamel adsorb phosphates at 40° in a manner comparable to the adsorption of phosphates by hydroxyapatite.

2. The adsorbing power of bone is greater than that of dentin which is greater than that of enamel. This order may depend on the fact that bone and dentin have smaller mineral crystals than are found in enamel and thus have larger adsorbing surfaces.

3. The importance of adsorption is indicated (*a*) in calcification phenomena and (*b*) in accounting for the presence of radioactive phosphorus in the surface of tooth enamel.

BIBLIOGRAPHY

1. Manly, M. L., and Levy, S. R., *J. Am. Chem Soc.*, **61**, 2588 (1939).
2. Crowell, C. D., Hodge, H. C., and Line, W. R., *J. Dent. Research*, **14**, 251 (1934).
3. Armstrong, W. D., *Proc. Soc. Exp. Biol. and Med.*, **44**, 28 (1940).
4. Bale, W. F, Haven, F. L., and LeFevre, M. L., *Rev. Scient. Instruments*, **10**, 193 (1939).
5. Manly, R. S., and Hodge, H C., *J. Dent Research*, **18**, 133 (1939).
6. Hodge, H. C., LeFevre, M. L., and Bale, W. F., *Ind. and Eng. Chem.*, *Anal. Ed.*, **10**, 156 (1938).
7. Volker, J. F., Hodge, H. C., Wilson, H. J., and Van Voorhis, S. N., *J. Biol. Chem*, **134**, 543 (1940).

8. Manly, R. S., Hodge, H. C., and Manly, M. L., *J. Biol. Chem.*, **134**, 293 (1940).
9. Manly, M. L., and Bale, W. F., *J. Biol. Chem.*, **129**, 125 (1939).
10. Manly, M. L., Hodge, H. C., and Van Voorhis, S. N., *Proc. Soc. Exp. Biol. and Med.*, **45**, 70 (1940).
11. Volker, J. F., and Sognnaes, R. F., *J. Dent. Research*, **19**, 292 (1940).
12. von Hevesy, G., and Armstrong, W. D , *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133**, p. xliv (1940).

.

CHOLINE IN THE NUTRITION OF CHICKS*

By D. MARK HEGSTED, R. C. MILLS, C. A. ELVEHJEM,
AND E. B. HART

(*From the Department of Biochemistry, College of Agriculture, University
of Wisconsin, Madison*)

The rôle of choline in the nutrition of the young rat has been studied extensively (1–4). Sure (5) has also shown the need of choline for lactation in mature animals. Its relation to the nutrition of the chick has not been studied, although Abbott and DeMasters (6) concluded that the addition of choline to a skim milk powder-polished rice ration increased egg production and decreased mortality in laying hens. More recently Jukes (7) has shown that turkey poults require choline for normal growth and the prevention of perosis.

In continuing the studies in this laboratory (8) on new factors required by growing chicks, we have encountered a severe choline deficiency. Chicks upon our basal ration show inferior growth and severe perosis which are entirely prevented by the addition of 0.1 per cent choline to the diet.

EXPERIMENTAL

Day-old white Leghorn chicks were divided into groups of six and placed in small heated brooders supplied with screen bottoms. The experimental ration and water were supplied immediately upon receipt of the chicks and were fed *ad libitum* thereafter. Individual weights were recorded at weekly intervals and the

occurrence of any symptoms noted. All experiments were terminated at 4 weeks, at which time the livers, bones, and blood of some of the chicks were taken for analysis.

The composition of the basal rations[1] used in these studies is shown in Table I. They are based upon Ration 470 reported previously (8) with additions of concentrates, yeast, or crystalline vitamins to supply the missing factors. Ration 473a contains in addition to cartilage (8) a well extracted kidney residue which

TABLE I

Composition of Rations

Constituent	Ration 473a	Ration 474
Dextrin	54	47
Casein, alcohol-extracted . .	18	18
Salts 4*...	5	5
Soy bean oil	5	5
Cartilage	15	15
Brewers' yeast . .		10
Kidney residue .	3	
Yeast eluate, % .	≅10	
Thiamine, *mg. per kg.* .	2	2
Riboflavin, " " " . .	2	2
Pyridoxine, " " " . .	2	2

Haliver oil fortified with vitamin D, 1 drop per chick per wk.

* Salts 4 is a modification of Salts 1 (Kline, O. L., Bird, H. R., Elvehjem, C. A., and Hart, E B., *J. Nutrition*, 11, 515 (1936)). The composition is

$CaCO_3$ . . .	600	$Fe(C_6H_5O_7)_2 \cdot 6H_2O$		55
K_2HPO_4 . .	... 645	KI. .	....	1.6
$CaHPO_4 \cdot 2H_2O$....	150	$MnSO_4 \cdot 4H_2O$.	..	10.0
$MgSO_4 \cdot 7H_2O$ . .	204	$ZnCl_2$	. . .	0.5
NaCl	335	$CuSO_4 \cdot 5H_2O$. .	. .	0 6

supplies an essential antidermatitis factor reported by Hegsted *et al.* (9) and a yeast eluate which supplies factor U (10, 11). This ration contains no appreciable amount of pantothenic acid

[1] The yeast used in these experiments was supplied by Dr. Harold F. Levine of the Pabst Brewing Company, Milwaukee; the haliver oil by Dr. C. Nielson of the Abbott Laboratories, North Chicago; the thiamine, pyridoxine, and pantothenic acid by Merck and Company, Inc., Rahway, New Jersey, the liver extract by The Wilson Laboratories, Chicago; and the soy bean oil by Allied Mills, Peoria, Illinois.

but this was supplied in the form of liver extract concentrates. The other ration (No. 474) contains brewers' yeast, which supplies all of these factors and perhaps others in adequate amounts at a 10 per cent level. Crystalline thiamine, riboflavin, and pyridoxine were added merely as a precaution against variation of these factors.

TABLE II

Growth-Promoting Activity of Liver Extract Fractions

Supplement to Ration 473a	No. of chicks	Average weight		No. dead at 4 wks.
		3 wks.	4 wks.	
		gm.	*gm.*	
None	6	50		6
4% liver extract	6	125	185	
Pantothenic acid concentrate ≅ 100 γ per day	11	84	102	
Acid ether extract ≅ 4% liver extract . .	6	67	78	1
Hexane-BuOH extract ≅ 6% liver extract .	6	78	88	
Acid ether " + hexane-BuOH extract ≅ 5% liver extract .	6	75	101	1
Acid ether extract + hexane-BuOH extract + hexane-BuOH residue ≅ 5% liver extract	6	129	178	
Acid ether extract + hexane-BuOH extract + acid ether residue ≅ 5% liver extract .	6	127	187	1
Acid acetone extract ≅ 5% liver extract	12	95	124	3
" " " ≅ 10% " "	6	120	144	
" " " + residue ≅ 3% liver extract	6	171	180	
Acid acetone extract + alcohol extract of acid acetone residue ≅ 6% liver extract .	6	185	201	
Norit filtrate ≅ 5% liver extract . . .	6	105	173	2
10% yeast	6	102	144	1

In Table II are shown typical results obtained upon fractionation of liver extract.[2] Although the addition of 4 per cent of liver extract resulted in fairly good growth, it is obvious that factors other than pantothenic acid were supplied, since pantothenic acid concentrates (12) allowed only very poor growth. Also, extracts from liver extract, which contain most of the pantothenic acid, did not contain appreciable amounts of the growth

[2] Wilson, Fraction D.

factor. The acid ether extract was made by dissolving 600 gm. of liver extract in 2 liters of water and adjusting the solution to pH 1 with HCl. The resulting precipitate was filtered off, washed with dilute acid, and the combined filtrates extracted continuously for 5 days with ether. Woolley *et al.* (13) have shown this extract to be a potent source of pantothenic acid. The hexane-butanol extract was made by the method of Black *et al.* (14). The liver extract was extracted by stirring with large volumes of butanol and an equal volume of hexane added to the combined extracts. After standing, the clear supernatant was concentrated for feeding. The method of Frost and Elvehjem (15) for the preparation of acid acetone extracts was used. A solution of 500 gm. of liver extract and 500 cc. of water was extracted four times with 4 volumes of acetone containing 0.5 cc. of concentrated HCl per liter. Uniformly poor growth was obtained with these extracts and in all cases the residues remaining after extraction were required to give satisfactory growth. The growth factor remaining in the residues was soluble in alcohol and not adsorbed upon norit from acid solution. Yeast proved to be a poor source of the factor, since 10 per cent did not support growth comparable to that obtained with 4 per cent of liver extract.

Since yeast contained little of the growth factor, it was possible to use a cruder ration (No. 474) for further investigations which offered less possibility of multiple deficiency. In Table III the growth obtained with this ration plus various supplements is shown. The number of chicks showing perosis in the various groups is also given. Additional cartilage or casein actually inhibited growth, while additional yeast gave only a slight response. 5 per cent of dry whole kidney gave excellent growth which was nearly equaled by the acid acetone residue and picric acid filtrate from liver extract. Liver extract heated for 36 hours at 120° was also effective. Finally the addition of choline at a 0.1 per cent level gave growth comparable to that obtained with liver extract fractions. In one run 0.2 per cent choline gave somewhat better growth.

Choline also prevents the occurrence of perosis upon this ration. Only one slight case has been noted in those groups receiving choline. Apparently 0.05 per cent of choline is sufficient to prevent perosis but is not enough to give maximum growth. This

probably explains the results obtained with fish-meal, when growth was not stimulated but perosis did not occur. It may also explain the failure of severe perosis to occur on Ration 473a, since the liver extract preparations probably contain small amounts of choline. In fact very severe perosis has been noted upon Ration 473a when crystalline pantothenic acid is added to the ration instead of liver extract concentrates.

TABLE III

Relation of Various Supplements and Choline to Growth and Perosis

Supplement to Ration 474	No. of chicks	Average weight		No. show-ing perosis	No. dead at 4 wks.
		3 wks.	4 wks		
		gm.	gm.		
None .	18	117	168	11	
12% additional casein	6	86	118	4	1
15% " cartilage	6	93	128	3	1
5% " yeast .	6	127	185		
12% fish-meal .	6	114	163		
30% yellow corn .	6	103	166	2	1
5% kidney .	6	159	234	1	
Acid acetone extract ≅ 4% liver extract	6	130	170	3	
" " residue ≅ 4% " "	6	173	214	1	
Picric acid filtrate ≅ 4% " "	6	150	213		
4% heated liver extract. .	5	168	210	1	
0.05% choline	5	134	180		
0.1% "	16	164	210	1	
0.2% " . .	6	189	232		
0.1% " *.	6	139	183		

* The food intake was limited to that eaten by the group on the basal diet.

That the action of choline in preventing perosis is not due to increased consumption of some unknown constituent of the ration was shown by limiting the food intake in one group receiving choline. Perosis was completely prevented, although the chicks gained no more than those receiving the basal ration alone.

The well known lipotropic action of choline led us to investigate the livers of these chicks. Autopsy revealed no gross abnormalities. Analysis of the livers of chicks receiving choline showed an average of 3.89 per cent of fat in the fresh tissue.

The livers of deficient chicks averaged 3.93 per cent of fat upon the same basis.

Since Wiese et al. (16) have shown a marked decrease in phosphatase content of the bones of perotic chicks on a manganese-deficient ration, the bone phosphatase of choline-deficient and choline-supplemented birds was investigated. Again no differences were found. The bones from perotic birds contained an average of 20.1 units per gm. of bone, while those receiving choline averaged 22.6 units. These values are somewhat higher than the value reported by Wiese for normal chicks of the same age.

<div align="center">DISCUSSION</div>

These results demonstrate the requirement of the growing chicks for rather large amounts of choline, approximately 0.1 per cent of the ration. Lower levels may suffice for the prevention of perosis. The requirement for turkey poults (7) and young rats (3, 4) has been reported to be about 0.3 per cent of the ration. These relatively high requirements may make choline an important factor in practical nutrition, although any decision must await further work upon distribution of choline in natural materials. It is of interest to note that although Fletcher et al. (17) report approximately 2.5 mg. of choline per gm. of yeast, analysis of the yeast in our rations failed to reveal the presence of choline.[3]

The livers of newly hatched chicks have been shown to contain large amounts of fat (18, 19). Tanzi (19) has also shown that the rate of removal of liver fat is markedly increased by the administration of choline. Our analyses show no accumulation of fat in the livers of choline-deficient chicks at 4 weeks of age. Thus the fat is eventually removed, even though the chick is maintained upon a choline-deficient ration.

Several investigators have indicated the possibility of an organic factor functioning in the prevention of perosis. Heller and Penquite (20) state that perosis is prevented by a heat-stable factor in water extracts of rice bran which was not replaceable by the ash of rice bran. Wiese et al. (21) found that autoclaved rice bran failed to protect chicks from perosis, while the untreated

---

[3] We wish to thank H. P. Jacobi for this analysis.

bran was effective. Later Hogan, Richardson, and Patrick (22) reported a factor present in 95 per cent alcoholic extracts of whole liver which in conjunction with manganese prevented perosis. Whether or not these results can be identified with the action of choline must await further investigation.

Perosis in chicks has usually been associated with rations high in calcium or phosphorus or both and low in manganese. None of these is characteristic of our ration. Indeed the salt mixture supplies almost a minimum amount of calcium, 0.6 per cent of the ration, and the calcium to phosphorus ratio of the ration containing 18 per cent of casein is about 1.38. According to Wiese et al. (16) 50 parts per million of manganese are sufficient to prevent perosis. The salt mixture used here supplies approximately 60 parts per million. Thus these two conditions are not concerned in perosis resulting from choline deficiency. Wiese and coworkers have also shown that a marked decrease in bone and blood phosphatase occurs in perotic birds upon high calcium and phosphorus rations and that this is prevented by manganese. This strongly indicates a relation between phosphatase and perosis. However, in choline deficiency no fall was noted. Thus the level of phosphatase is not concerned, although it is possible that the function of phosphatase may be impaired in choline deficiency.

SUMMARY

Choline has been shown to be essential for growth and the prevention of perosis in growing chicks. Choline-deficient chicks did not show fatty livers at 4 weeks of age and bone phosphatase values were normal in chicks suffering from perosis due to choline deficiency.

BIBLIOGRAPHY

1. Best, C. H , and Ridout, J. H., in Luck, J. M., Annual review of biochemistry, Stanford University, 8, 349 (1939)
2. Channon, H. J., in Luck, J. M., Annual review of biochemistry, Stanford University, 9, 231 (1940).
3. Griffith, W. H., and Wade, N. J., J. Biol. Chem., 131, 567 (1939).
4. György, P., and Goldblatt, H., J. Exp. Med., 72, 1 (1940).

5. Sure, B., *J. Nutrition*, **19,** 71 (1940).
6. Abbott, O. D., and DeMasters, C. U., *J. Nutrition*, **19,** 47 (1940).
7. Jukes, T., *J. Biol. Chem.*, **134,** 789 (1940).
8. Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Poultry Sc.*, **19,** 167 (1940).
9. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **20,** 599 (1940).
10. Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **125,** 687 (1938).
11. Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133,** p. xli (1940).
12. Oleson, J. J., Woolley, D. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **42,** 151 (1939).
13. Woolley, D. W., Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Biol. Chem.*, **125,** 715 (1938).
14. Black, S., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, **132,** 65 (1940).
15. Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, **121,** 255 (1937).
16. Wiese, A. C, Johnson, B. C., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, **127,** 411 (1939).
17. Fletcher, J. P, Best, C. H., and Solandt, O. M., *Biochem. J.*, **29,** 2278 (1935).
18. Entenman, C., Lorenz, F. W., and Chaikoff, I. L , *J. Biol. Chem.*, **133,** 231 (1940).
19. Tanzi, B., *Boll. Soc. ital. biol. sper.*, **13,** 466 (1938).
20. Heller, V. G., and Penquite, P., *Poultry Sc.*, **15,** 425 (1936).
21. Wiese, A. C., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *Poultry Sc.*, **17,** 33 (1938).
22. Hogan, A. G., Richardson, L. R., and Patrick, H., *Proc. Am. Inst. Nutrition*, *J. Nutrition*, **19,** 14 proc. (1940).

METHIONINE IN THE DIET OF THE CHICK

BY A. A. KLOSE AND H. J. ALMQUIST

(From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley)

Workers in this laboratory (1–3) have shown that the requirements for amino acids in the nutrition of the chick do not necessarily correspond with those of the rat, particularly in the cases of arginine and glycine. It seems necessary to determine whether further differences in amino acid requirements exist between the chick and the rat. In the work to be reported here, the essential nature of methionine and its replaceability by cystine and homocystine have been investigated with an arachin diet.

The arachin was prepared from peanut meal according to the method of Johns and Jones (4). The methionine, cystine, and choline were commercial products. The homocystine was synthesized by the method of Patterson and du Vigneaud (5).

The experimental procedure was similar to that previously described (1). Newly hatched, single comb white Leghorn chicks were placed on a standard chick mash for 1 week, then weighed, wing-banded, divided into groups, and placed on the experimental diets. Each group contained six chicks of the same average weight and weight distribution. The individual chick weights were recorded at 1 or 2 day intervals.

The basal diet for Experiments I and II consisted of arachin 20 per cent, gelatin 5, gum arabic 5, cod liver oil 1, dried alfalfa 1, sodium chloride (containing traces of other elements) 1, calcium carbonate 1, tricalcium phosphate 3, *l*-tryptophane 0.1, brewers' yeast 5, Galen B (rice bran extract) 3, and cerelose (dextrose) 54. The basal diet for Experiment III was the same except for these differences: brewers' yeast 7, Galen B 0.5, substitution of a 1:1 starch-sucrose mixture for the cerelose, and addition of 0.075 per cent *dl*-threonine.

467

From the results presented in Table I it is evident that the basal diet was decidedly inadequate for the chick with respect to its methionine content. A supplement of at least 1 per cent *dl*-methionine was necessary for optimum growth. Apparently *l*-cystine could not effectively replace methionine as far as growth-promoting effect is concerned.

TABLE I

Growth-Promoting Effects of Supplements in Methionine-Deficient Diet for Chicks

Experiment No.	Group No.	Supplement to diet	Level	Interval on diet	Per cent gain per day*
			per cent	*days*	
I	1	None		8	−1.08
	2	*dl*-Methionine	0.50	8	+3.44
	3	"	0.75	8	+4.43
	4	*l*-Cystine	1.00	8	+0.07
II	1	None		7	−0.59
	2	*dl*-Methionine	0.75	7	+4.09
	3	"	1 00	7	+4.59
III	1	Choline chloride	0 2	1–10	−0 26
	2	*dl*-Homocystine	0.9	1–10	+1.45
	3	*dl*-Methionine	1 0	1–10	+5.82
		Choline chloride	0.2		
	4	None		1– 5	−0.46
	4	*dl*-Methionine	1.0	6–10	+6 30
	5	*dl*-Homocystine	0 9	1– 5	+3.51
		Choline chloride	0.2		
	5	*dl*-Homocystine	0.9	6–10	+6.36
		Choline chloride	0 5		

* Weight increase in gm. per 100 gm. of body weight.

Experiment III was designed to test the ability of homocystine, with and without choline, to replace methionine in this diet. The results indicate that homocystine can effectively replace methionine only when a sufficient amount of choline is present. An addition of 0.2 per cent of choline chloride did not allow complete utilization of the homocystine, but 0.5 per cent led to growth equivalent to that obtained on the 1 per cent methionine diets. The level of choline necessary to insure efficient utilization of homocystine is comparatively high. On the other hand, the

basal ration appears to have sufficient choline for good growth when supplemented with methionine alone.

The percentage gains of groups fed homocystine show a linear relation to the added choline chloride. If this relation is assumed to hold at lower levels of choline, it may be estimated that the equivalent of 0.15 per cent of choline chloride was present in the basal ration or in the chicks or both. While such a calculation is by no means proved to be valid at the present time, it is interesting to note that Jukes (6) has reported that choline is a growth factor for the chick and that a level of 0.1 per cent of added choline chloride was optimum for chicks on his diets.

In a subsequent test we added creatine to the basal diet plus choline. No growth-promoting effect was found. Creatine evidently cannot replace methionine in these diets.

The above results indicate that the choline-homocystine-methionine relation established for the rat by du Vigneaud, Chandler, Moyer, and Keppel (7) holds true for the chick as well.

SUMMARY

1. Methionine is essential for the growth of the chick.

2. Neither cystine nor homocystine alone can replace methionine in its growth-promoting effect. Creatine did not replace methionine.

3. Homocystine and choline together can effectively replace methionine in the diet of the chick.

BIBLIOGRAPHY

1. Klose, A. A., Stokstad, E. L. R., and Almquist, H. J., *J. Biol. Chem.*, **123**, 691 (1938).
2. Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, **134**, 213 (1940).
3. Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, **135**, 153 (1940).
4 Johns, C. O., and Jones, D. B., *J. Biol. Chem*, **28**, 77 (1916-17).
5. Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, **111**, 393 (1935).
6. Jukes, T. H., *J. Nutrition*, **20**, 445 (1940).
7. du Vigneaud, V, Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J Biol. Chem.*, **131**, 57 (1939).

THE B VITAMINS AND FAT METABOLISM

IV. THE SYNTHESIS OF FAT FROM PROTEIN

BY E. W. McHENRY AND GERTRUDE GAVIN

*(From the Department of Physiological Hygiene, School of Hygiene,
University of Toronto, Toronto, Canada)*

It has been shown by us (1, 2) that in rats and in pigeons thiamine is required for the synthesis of fat from carbohydrate. Longenecker (3) has reported the synthesis of fat from protein in rats fed a diet containing casein, salts, and yeast. Hoagland and Snider (4) have confirmed Longenecker's results. In both cases yeast was supplied in the diet and it seemed desirable to determine whether thiamine or some other factor in the yeast was responsible for the synthesis of fat from protein.

Methods

White rats of the Wistar strain, reared in the Connaught Laboratories' colony, were employed. The general care of the animals has been reported previously (5). They were depleted of their body stores of the B vitamins and the body fat was reduced to a low level by placing them for 3 weeks on Basal Diet 1 (5). The rats were then fed Basal Diet 3, with the following composition in per cent by weight: casein (Labco) 96, salts mixture (Steenbock-Nelson Salts 40 (6)) 4, cod liver oil concentrate 0.015. During the period of protein feeding, vitamin and choline supplements were administered, by subcutaneous injection, in the following amounts per rat per day, unless otherwise indicated: thiamine hydrochloride (Merck) 20 γ, riboflavin (Hoffmann-La Roche) 20 γ, pyridoxine 40 γ, calcium pantothenate 100 γ, nicotinic acid (Eastman Kodak) 0.1 mg., choline hydrochloride (British Drug Houses) calculated as choline base 10 mg. We are indebted to Merck and Company, Inc., for gifts of pyridoxine and calcium pantothenate.

The rats were killed by stunning, the livers removed, and the total crude fatty acids in the livers and bodies were determined by methods previously published (1, 7).

A number of experiments have been carried out.   Since the results have been in good agreement, two typical experiments will be described.
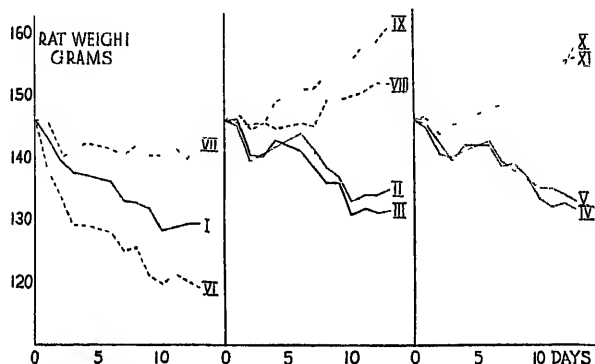
Fig. 1. The effect of the B vitamins upon the body weight of rats fed a high protein diet.   Supplements: Curve I, thiamine; Curve II, thiamine, riboflavin; Curve III, thiamine, riboflavin, calcium pantothenate; Curve IV, thiamine, riboflavin, calcium pantothenate, nicotinic acid; Curve V, thiamine, riboflavin, calcium pantothenate, nicotinic acid, choline; Curve VI, pyridoxine; Curve VII, pyridoxine, thiamine; Curve VIII, pyridoxine, thiamine, riboflavin; Curve IX, pyridoxine, thiamine, riboflavin, calcium pantothenate; Curve X, pyridoxine, thiamine, riboflavin, calcium pantothenate, nicotinic acid; Curve XI, pyridoxine, thiamine, riboflavin, calcium pantothenate, nicotinic acid, choline.

Series I—The effect of thiamine administered alone, and in various combinations with the other known factors of the vitamin B complex, was investigated.   The body weight changes are shown in Fig. 1.   The total crude fatty acids in the livers and bodies are given in Table I.   All results are averages for nine rats.

Series II—Two groups, each of fifteen rats, received Basal Diet 3, thiamine, riboflavin, nicotinic acid, calcium pantothenate, and choline during the entire experiment.   Group B was given, in addition, 20 γ of pyridoxine per rat per day.   After 5 weeks

ten rats in each group were killed. At this time the average weight of the rats receiving pyridoxine was 30 gm. greater than that of the deficient animals in Group A.

The remaining five rats in Group B were continued on the same régime for 3 weeks longer. During this period the residual five rats in Group A were given 40 γ of pyridoxine daily. This caused

TABLE I

Effect of B Vitamins upon Liver and Body Fat of Rats Fed High Protein Diet

Series No.	Group	Supplements						Total crude fatty acids		
		Thia-mine	Ribo-flavin	Panto-thenic acid	Nico-tinic acid	Cho-line	Pyrid-oxine	Liver	Body	
								per cent	per cent	gm.
I								7.2	3.2	4.7
		+						5.3	2.4	3.1
		+	+					7.0	2.7	3.6
		+	+	+				9.2	2.4	3.2
		+	+	+	+			8.2	2.3	3.0
		+	+	+	+	+		5.6	2.2	2.9
							+	2.0	1.8	2.1
		+					+	2.1	3.4	4.8
		+	+				+	2.0	4.2	6.4
		+	+	+			+	3.1	5.7	9.2
		+	+	+	+		+	3.1	5.6	8.9
		+	+	+	+	+	+	2.7	5.4	8.4
II	A*	+	+	+	+	+		2.9	3.5	4.4
	B*	+	+	+	+	+	+	2.2	5.8	9.3
	A†	+	+	+	+	+	+‡	3.7	9.7	14.0
	B†	+	+	+	+	+	+	2.6	10.5	15.5

* After 5 weeks.

† After 8 weeks.

‡ Pyridoxine was supplied during the last 3 weeks of the experiment.

a marked increase in body weight, so that at the end of the experiment the average weight of the rats in Group A was only 3 gm. less than that of the rats in Group B. The total crude fatty acids of the livers and bodies are given in Table I.

DISCUSSION

In the absence of pyridoxine there was a steady loss of weight in rats fed a diet high in protein but devoid of fat and carbohydrate, regardless of the administration of any of the other known

B vitamins. Pyridoxine administered with thiamine prevented weight loss.   The further addition of pantothenic acid and ribo-flavin resulted in a gain in weight which was not augmented by nicotinic acid or choline.   The necessity of pyridoxine for any gain in weight of rats fed a protein diet is in marked contrast to its slight activity when the diet is high in carbohydrate (5).

We have previously reported that pyridoxine would not prevent the deposition of fat in the liver which resulted when thiamine was administered to rats receiving a high carbohydrate diet (5). In the present experiments, in which the rats received a protein diet free of carbohydrate, a lower value for liver fat was obtained in all cases when pyridoxine was given.   Under these conditions pyridoxine has the lipotropic effect which was reported by Hal-liday (8).

The administration of thiamine alone or in various combinations with riboflavin, pantothenic acid, nicotinic acid, and choline in all cases resulted in a lower value for body fat than that of the con-trol group.   The control rats were killed at the end of the defi-ciency period; therefore they had received neither the protein diet nor any of the B vitamin supplements.   Choline was included in these tests in order that the body could handle fat normally and to prevent the possibility that fat synthesis might be impeded by accumulation of fat in the liver.   Administration of all these known substances failed to give fat synthesis from protein.

A supply of pyridoxine alone resulted in the greatest loss of body fat, but if thiamine was also given the same amount of body fat was secured as in the control.   This would indicate either that no fat had been burned by the animal during the 2 week period or that fat was being slowly synthesized from protein and was being used up in metabolic processes.

Pyridoxine, thiamine, riboflavin, and pantothenic acid admin-istered together resulted in the greatest value for body fat, about double the value of the control.   Approximately 30 per cent of the increase in body weight can be accounted for by increase in fat content.   This clearly indicates fat synthesis which was not possible when these vitamins were given in the absence of pyri-doxine.   The further addition of nicotinic acid and choline did not increase the body fat.

Rats fed the protein diet and receiving supplements of thiamine,

riboflavin, pantothenic acid, nicotinic acid, choline, and pyridoxine gained weight and after 5 weeks the level of body fat was double that of a group receiving the same treatment except for the omission of pyridoxine. When these deficient animals were given pyridoxine, there was a fairly rapid increase in body weight and the amount of body fat was tripled in 3 weeks. This confirms the fact that pyridoxine is required for the synthesis of fat when the diet contains only protein.

No information is available to indicate the nature of the mechanism by which pyridoxine brings about fat synthesis from protein. As a hypothesis it is suggested that pyridoxine is necessary for such stages of protein metabolism as may be essential for the formation of carbohydrate. The previously demonstrated action of thiamine in promoting fat synthesis from carbohydrate would explain why pyridoxine alone has been found to have no effect in these experiments.

SUMMARY

While the administration of thiamine alone will cause the synthesis of fat from carbohydrate, it will not do so from protein. In the latter case pyridoxine is essential. This vitamin, in combination with thiamine, prevents a reduction in body fat of rats maintained on a protein diet. The synthesis of fat from protein is clearly evident when pyridoxine, thiamine, riboflavin, and pantothenic acid are supplied. Various combinations of the isolated members of the vitamin B complex do not cause the synthesis of fat from protein unless pyridoxine is present. It is suggested that pyridoxine is essential for the metabolism of protein.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
2. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **128**, 45 (1939).
3. Longenecker, H. E., *J. Biol. Chem*, **128**, 645 (1939).
4. Hoagland, R., and Snider, G. G., *J. Nutrition*, **18**, 435 (1939).
5. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
6. Steenbock, H, and Nelson, E. M., *J. Biol. Chem*, **56**, 362 (1923).
7. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
8. Halliday, N., *J. Nutrition*, **16**, 285 (1938).

IS CHOLINE THE FACTOR IN THE PANCREAS THAT PREVENTS FATTY LIVERS IN DEPANCREATIZED DOGS MAINTAINED WITH INSULIN?

By C. ENTENMAN and I. L. CHAIKOFF

(*From the Division of Physiology, University of California Medical School, Berkeley*)

It has been known for some time that the feeding of raw pancreas prevents the development of fatty livers in completely depancreatized dogs maintained with insulin. Recent developments have dealt with two phases of this problem, (1) the mechanism by which the gland eliminates the anti-fatty liver factor and (2) the substance in the gland that is effective in this respect. The fact that fatty livers result from the exclusion of the external secretion of the pancreas from the gastrointestinal tract by duct ligation (1–3), as well as the observation that the daily feeding of fresh pancreatic juice inhibits the deposition of excessive amounts of fat under these conditions (4), suggests that the release of pancreatic juice into the gastrointestinal tract under normal conditions serves to maintain a normal lipid content in the liver. The finding that choline is just as effective as the raw glandular tissue in preventing fatty livers from occurring in duct-ligated (5) and in depancreatized dogs maintained with insulin (6, 7) led to the belief that choline was the active constituent in the pancreas. Dragstedt and his coworkers, however, claim to have ruled out choline (8). As evidence for this view they point to their observation that the daily administration of 200 mg. to 1 gm. of choline for 9 to 28 days failed to cure fatty livers in completely depancreatized dogs (weighing 9.4 to 14.5 kilos) maintained with insulin. But a measurable effect of choline need not have been expected under the conditions of their experiment. It was shown elsewhere that, although the *preventive* action of choline can be readily demonstrated when daily feedings of it are instituted imme-

diately after pancreatectomy, its *curative* action is very slow, daily feedings of as much as 3 gm. daily being necessary to produce measurable effects upon livers in which large amounts of fat have already accumulated (7). Thus 26 per cent fatty acids were found in a liver of a 6.3 kilo dog despite the ingestion of 2 gm. of choline chloride daily during the 6 weeks preceding removal of its liver. Moreover, the feeding of the same amount of choline to dogs weighing 5.8 to 8.0 kilos for 11.5 to 15 weeks failed to restore the lipid content of the liver to normal. Curiously enough, the entire series of observations on choline reported by Dragstedt *et al.* consisted of those on four dogs, two of which died 4 to 5 weeks after pancreatectomy despite the fact that, as shown in this laboratory (9), such animals can be kept alive for years without choline supplements when maintained on a diet of lean meat and sucrose, supplemented with vitamins and salts. More recently these workers claimed to have observed effects with amounts of a pancreas fraction as small as 55 to 125 mg. (10). It must be clear, however, from the above considerations that further investigations of the rôle of choline in the total physiological action of raw pancreas are highly desirable. It is still an open question whether the lipotropic effect of raw pancreas in the depancreatized dog is the result of its choline content or whether it contains a liver factor in addition to the choline.

Although the above discussion has dealt with the fatty livers of only the completely depancreatized dog, it should be noted here that the lipotropic effects of choline and pancreas have also been compared in the case of fatty livers produced in *normal rats* by the feeding of a *low protein-high fat diet*. No agreement as to whether choline accounts for the total lipotropic effect of the pancreas has been obtained even in the rat (11–13). It appears necessary to point out again, however, that, since the mechanisms for the production or cure of fatty livers in the *depancreatized dog* and *normal rat* have not been shown to be identical, conclusions derived from one should not at present be applied to the other.

EXPERIMENTAL

Twenty-five adult dogs were used in the present study. For periods of 1 to 3 weeks before pancreatectomy, each dog was fed per day a mixture containing 30 gm. of lean meat and 6 gm. of

sucrose per kilo.   Vitamins[1] and salts were added to this mixture daily in the form of 3 cc. of cod liver oil, 5 cc. of galen B (vitab, Type II, liquid), and 2 gm. of Cowgill's salt mixture (14).

The standard diet fed twice daily after pancreatectomy consisted of 250 gm. of lean meat, 50 gm. of sucrose, and 5 gm. of bone ash.   The vitamin supplements recorded above were added once daily.   Food was administered at 8.00 a.m. and 4.00 p.m.; 8 units of insulin[2] were injected at each time of feeding.

In the present investigation various amounts of choline and of two pancreas fractions (AR and KAR) were examined with respect to their effects in inhibiting fat deposition in the livers of depancreatized dogs.   The feedings of these substances were not begun immediately after pancreatectomy; they were begun when the animals had fully recovered from the operation, as shown by complete healing of the wound and by the acquisition of a vigorous appetite.   During the interval between the operation and the commencement of the administrations of choline, Fraction AR, and Fraction KAR, each depancreatized dog received 125 gm. of raw pancreas twice daily in addition to the standard mixture listed above.   This precaution was believed to insure the presence of a normal lipid content in the liver (15) at the time that choline, Fraction AR, and Fraction KAR treatments were started.

Livers were removed for analysis between 8.00 and 9.00 a.m. after the animals had been anesthetized with an intravenous injection of nembutal.   At this time the dogs were in the postabsorptive state, having received their last meal and injection of insulin at about 4.00 p.m. of the previous day.   The whole liver was thoroughly ground and the lipids determined from 10 gm. samples by the oxidative procedures described elsewhere (16).   Free cholesterol was determined in an acetone solution after the phospholipid had been precipitated.

Minimum Dose of Choline Necessary to Prevent Fatty Livers

It must be apparent by now that no decision concerning the relation of choline to the action of pancreas upon the livers of the

---

[1] The standardized cod liver oil was kindly furnished by Mead Johnson and Company; the vitamin B concentrate by Vitab Products, Inc., Emeryville, California.

[2] The insulin used in this study was generously supplied by Eli Lilly and Company.

completely depancreatized dog receiving insulin can be made until the minimum effective dose of choline is known. The effects of various amounts of choline upon the lipid content of the livers of

TABLE I

On Minimum Amount of Choline Required to Prevent Fatty Livers

The liver values are expressed as per cent of wet weight of tissue.

Dog No.	Weight			Period of observation*			Choline			Liver				
	Preoperative	Choline added	Final	Total	Receiving pancreas	Receiving choline	Per day	Per kilo of preoperative weight	Per kilo when started	Weight	Cholesterol		Total fatty acids	Phospholipid
											Total	Free		
	kg.	kg.	kg.	wks.	wks.	wks.	mg	mg.	mg.	gm.	per cent	per cent	per cent	per cent
D-183	6.0	6.5	7 6	26	5	21	75	13	12	400			17.00	
D-235	6 6	6.6	5.3	23	7	16	100	15	15	415	0 33		13 30	1 28
D-240	9.8	9.8	7.1	20	3	17	100	10	10	360	0.54	0.21	16 80	1.71
D-242	9.0	8.3	5.0	20	3	17	100	11	12	235	0.33	0.19	10 60	1.98
D-186	11.8	10.4	9.5	23	3	20	150	13	15	455			3.89	
D-215	8 8	8.4	5 3	22	1	21	300	34	36	270	0.21	0 16	2 57	1.88
D-221	6.9	7.7	6.7	19	4	15	300	44	39	315	0.30	0 20	2.38	
D-220	6.6	7.0	6.0	23	4	19	300	46	43	377	0 28	0 18	2.16	1 40
D-181	11.2	11.2	8 8	22	2	20	400	36	36	490	0 24	0.24	2.22	1.90
D-214	11.5	11 0	6.7	6	1	5	400	35	36	260	0.31	0.28	2.35	1.90
D-163	10.3	9.8	7.2	6	2	4	400	39	41	355	0 32	0.32	6.45	1.60
D-175	8.0	7.5	5.0	24	3	21	400	50	54	340	0 30	0.28	3.03	1.73
D-227	9.5	9.3	7.4	28	8	20	500	53	54	320	0.35		4.50	1 15
D-151	10.5	10.0	7.6	28	6	22	600	57	60	340	0.25	0.25	2 65	1.80
D-156	11.3	10.0	8.0	13	2	11	600	53	60	360	0.30	0.30	2.37	1 96
D-145	7.5	7.5	8.0	22	3	19	800	107	107	390	0.21	0.20	3.52	1.78
D-154	14.5	11.7	7.0	27	5	22	800	56	69	365	0.21	0.21	2.50	1.23
D-164	10.3	10 0	3.8	13	5	8	800	78	80	140	0.28		2.35	2.35

* Each dog after pancreatectomy received twice daily 250 gm. of meat, 50 gm. of sucrose, 5 gm. of bone ash, and 125 gm. of raw pancreas. Vitamin supplements were given twice weekly. When the dogs regained their appetites, the raw pancreas was eliminated from their diets and the amounts of choline specified above added to their evening meals.

eighteen depancreatized dogs were therefore determined; the results are recorded in Table I. The administrations of choline were not begun until intervals of 1 to 8 weeks had elapsed after excision of the pancreas. As noted above, the lipid content of

the liver was normal at the time choline treatments were introduced (7).

With few exceptions, choline was fed for 15 to 22 weeks. It was previously shown that this period is required for fatty livers to develop consistently in depancreatized dogs maintained with insulin (15).

The effect of 75 mg. of choline was tested in a single dog, No. D-183. This amount of choline was fed daily for 21 weeks; at the end of this period the dog's liver contained 17 per cent fatty acids. Three dogs received 100 mg. of choline daily for 16 to 17 weeks; their livers contained 11 to 17 per cent fatty acids.

Complete prevention of fatty livers was observed when doses of 36 mg. or more choline per kilo of body weight were employed. Six dogs received choline in amounts varying from 36 to 43 mg. per kilo. Whether examined at the end of 4 weeks or at the end of 21 weeks after the daily administrations of this amount of choline had been begun, the lipid content of the liver was normal or quite close to normal. Thus the total fatty acid content of the livers of five of the six dogs did not exceed 2.6 per cent. In a single dog, No. D-163, the total fatty acid content of the liver was 6.4 per cent. In seven other animals the dose of choline varied from 54 to 107 mg. per kilo daily. These doses were also effective in inhibiting the deposition of excessive amounts of lipid in the liver.

Effect of Small Amounts of Pancreas Fractions upon Lipid Content of Liver

In the experiments described below, the whole gland was *not* used. A fraction of pancreas designated as Fraction AR was tested. It was prepared as follows: The raw glandular tissue obtained fresh from the slaughter-house was ground and thoroughly shaken with 2 volumes of acetone for 1 hour. The residue was extracted for a second time with the same amount of acetone and then pressed to remove as much of the solvent as possible. The residue was then dried in warm air, ground to a fine powder, and finally extracted with ethyl ether in a Soxhlet apparatus for 8 hours. This residue has been designated Fraction AR. It was stored at −18° until used. 1 gm. of this pancreas fraction corresponds to approximately 5.5 gm. of the original glandular tissue.

Table II shows that supplementing the standard diet with as small an amount of Fraction AR as 1 gm. per day is sufficient to prevent completely the development of fatty livers. In all three dogs the livers were analyzed for lipids after administration of Fraction AR had been continued for a full 20 weeks.

In another group of dogs, the effect of heat on the anti-fatty liver action of Fraction AR was examined. Fraction KAR was

TABLE II

Effect of Daily Ingestion of Pancreas Fractions on Liver Lipids

The liver values are expressed as per cent of wet tissue.

Dog No.	Weight		Period of observation*		Pancreas fraction			Liver				
	Preoperative	Final	Total time	Period fed raw pancreas	Type	Period fed	Amount fed	Weight	Cholesterol		Total fatty acids	Phospholipid
									Total	Free		
	kg.	kg.	wks.	wks.		wks.	gm.	gm.	per cent	per cent	per cent	per cent
D-231	6.7	6.0	24	4	AR	20	1	460	0.21	0.13	2 26	1.44
D-233	5.9	6.7	24	4	"	20	1	370	0.23	0.16	1 65	2 11
D-238	8 0	7.4	23	3	"	20	1	450	0.17	0.18	3.78	2 18
D-264	13 0	8 8	22	5	KAR	17	4	744	0.33	0 14	31.80	1.23
D-265	11.1	8.8	22	5	"	17	4	565	0.30	0.19	23.80	1.32
D-267	7.2	7 1	22	5	"	17	4	283	0.44	0.38	18.20	3.46

* Each dog after pancreatectomy received twice daily 250 gm. of meat, 50 gm. of sucrose, 5 gm. of bone ash, and 125 gm. of raw pancreas. Vitamin supplements were given twice weekly. When the dogs regained their appetites, the raw pancreas was eliminated from their diets and the amounts of Fractions AR and KAR specified above added to their evening meals. For the meaning of Fractions AR and KAR see the text.

employed, which was prepared by subjecting Fraction AR to 20 pounds of steam pressure in an autoclave for 30 minutes. The daily administration of Fraction KAR in amounts equal to 4 times the dose found effective for Fraction AR failed to inhibit the development of fatty livers. These results show quite conclusively that the anti-fatty liver substance in Fraction AR is completely destroyed by heat.

DISCUSSION

The present investigation shows that 36 mg. of choline per kilo per day or 300 mg. for an 8.4 kilo dog are sufficient to inhibit completely the deposition of abnormal amounts of lipids in the livers of depancreatized dogs maintained with insulin. This amount of choline was found effective in keeping the fatty acid contents of the liver at 3 to 4 per cent levels even when the period of observation was extended to 5 months. Although this may not be the smallest effective dose of choline, it is nevertheless definitely established here that a daily dose as small as 15 mg. per kilo per day will not prevent the accumulation of excessive amounts of fat in the liver.

In earlier studies the feeding of 250 gm. of raw pancreas daily was adopted as a safe procedure for preventing fatty livers in depancreatized dogs (15). The same amount was employed here in the interval between the operation and the introduction of the administration of choline or pancreas fraction. This quantity of raw pancreas contains about 575 mg. of choline (17). It is now clear that, in the case of the ingestion of such quantities of raw pancreas, its choline content is sufficient to account for the observed lipotropic effect. Its choline content may also explain the preventive as well as curative action previously obtained with 250 gm. of autoclaved pancreas (15). Choline is, in all probability, the heat-stable factor referred to elsewhere (15).

The observation that the daily ingestion of 1 gm. of Fraction AR will completely prevent fatty livers in depancreatized dogs shows that the choline *present in this pancreatic fraction* is not its active agent. At most, 1 gm. of Fraction AR could contain 13 mg. of choline;[3] as noted above, 100 mg. of choline did not keep the liver normal in its fat content (Table I). The destruction of the active principle in Fraction AR by heat, as shown by the experiments in which Fraction KAR was fed, also suggests that the choline present in Fraction AR is not involved. It should be stressed, however, that while the lipotropic action of 1 gm. of Fraction AR

[3] This estimate is based on the choline content of whole raw pancreas (17)   The actual choline content of Fraction AR is probably less than 13 mg per gm , for some of the choline is removed by the acetone-ether treatment used in its preparation.

cannot be ascribed to its choline content, the possibility has not been ruled out that additional amounts of choline are made available to the organism by the presence of Fraction AR in the gastrointestinal tract.

SUMMARY

The lipotropic effects of choline and pancreas were compared in the completely depancreatized dog maintained with insulin.

1. It is shown that 35 mg. of choline per kilo are sufficient for complete prevention of fatty livers.

2. The daily feeding of a fraction derived from 5.5 gm. of raw pancreas completely inhibited the development of fatty livers even when fed for as long as 5 months.

3. The choline content of the pancreas fraction did not account for its lipotropic effect.   The possibility that choline is made available to the organism by the presence of the fraction in the gastrointestinal tract has not been ruled out.

4. The anti-fatty liver factor of the pancreas is heat-labile.

BIBLIOGRAPHY

1. Aubertin, E., Lacoste, A., and Castagnou, R., *Compt. rend. Soc. biol.*, **118**, 149 (1935).
2. Ralli, E. P., Rubin, S. H., and Present, C. H., *Am. J. Physiol.*, **122**, 43 (1938).
3. Montgomery, M. L., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **128**, 387 (1939).
4. Montgomery, M. L., Entenman, C., Chaikoff, I. L., and Nelson, C., *J. Biol. Chem.*, **137**, 693 (1941).
5. Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **135**, 329 (1940).
6. Best, C. H., Ferguson, G. C., and Hershey, J. M., *J. Physiol.*, **79**, 94 (1933).
7. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **120**, 647 (1937).
8. Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, **117**, 166 (1936).
9. Chaikoff, I. L., and Kaplan, A., *J. Nutrition*, **14**, 459 (1937).
10. Clark, D. E., Vermeulen, C. W., Donovan, P. B., and Dragstedt, L. R., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **126**, P464 (1939).

11. Aylward, F. X., and Holt, L. E., Jr., *J. Biol. Chem.*, **121**, 61 (1937).
12. Best, C. H., and Ridout, J. H., *Am. J. Physiol.*, **122**, 67 (1938).
13. Channon, H. J., Loach, J. V., and Tristram, G. R., *Biochem. J.*, **32**, 1332 (1938).
14. Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).
15. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **119**, 435 (1937).
16. Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **123**, 577 (1938).
17. Fletcher, J. P., Best, C. H., and Solandt, O. M., *Biochem. J.*, **29**, 2278 (1935).

CRYSTALLINE INSULIN OF LOW ZINC CONTENT

By MELVILLE SAHYUN

(From the Biochemical Research Laboratory, Frederick Stearns and Company, Detroit)

Cohn and coworkers (1) reported the isolation of a crystalline insulin containing 0.36 per cent zinc. This was interesting, since Scott (2) had previously reported that crystalline zinc insulin contains 0.52 per cent zinc. Recently Maxwell and Feldkamp (3) confirmed the findings of Cohn and his coworkers.

In June, 1936, I prepared a crystalline insulin, containing considerably less zinc than any reported in the literature, without the use of zinc other than that present in the pancreas It was crystallized directly from the first salt cake of a pancreatic extract by dissolving the crude insulin in 0 05 M potassium acid phosphate and 10 per cent isopropyl alcohol with 1 N hydrochloric acid The concentration of insulin was about 30 units per cc. The acidity was then adjusted to about pH 6.8 and the mixture was warmed to about 50°. A heavy precipitate formed was removed by filtration. The acidity of the clear filtrate was brought to about pH 6.5. Cooling was allowed to proceed very slowly overnight, whereupon crystals were formed. The filtrate was then placed in a refrigerator for several days. The crystals which were recovered were oval in shape and were termed egg-shaped crystals (Fig 1).

Recrystallization—5 gm of the egg-shaped crystals were suspended in about 500 cc. of distilled water and 200 cc. of 0.5 M potassium acid phosphate, pH 4.5 Dilute 1 N lactic acid was added, 1 cc. at a time, until the crystals were completely dissolved 200 cc of isopropyl alcohol and enough distilled water were added to bring about a volume of 1900 cc. The mixture was thoroughly stirred and 1 N ammonium hydroxide was added, 1 cc at a time, until pH 6.6 to 6.7 was reached Filtered steam

was next passed into the mixture until the temperature of the liquid was approximately 50°. Insoluble matter that formed was removed by filtration through a clarifying Seitz pad. The filtrate, which was clear and almost colorless, was diluted to 2000 cc., brought to pH 6 5 with 1 N lactic acid, stirred thoroughly, and allowed to cool at room temperature. In about an hour crystals developed. The mixture was allowed to stand overnight at room temperature. As the insulin crystallized, the mother liquor became more alkaline. The pH was accordingly adjusted to exactly 6.5 with 1 N lactic acid, with a glass electrode, and the

Fig 1   Fig. 2

Fig. 1. Egg-shaped insulin crystals. × 222
Fig. 2. Recrystallized insulin of low zinc content.

suspension was placed in a refrigerator at 2–4°. After 2 days the crystals were recovered by centrifugation, washed twice with distilled water in the centrifuge bottle, and a third time with warm (45°) distilled water. After the third washing the crystals were suspended in a small quantity of distilled water and filtered through a small Buchner funnel, and then washed successively with 50 per cent, 70 per cent, and 95 per cent ethyl alcohol, finally with acetone, and then dried in a vacuum. The yield was 3 086 gm.

The crystals that were thus obtained were fairly uniform in size and belonged to the rhombohedral type as shown in Fig 2. As it may be noted, the edges of the crystals are not very sharp.

This may be due to first, concentration and second, rapidity of crystallization. Subsequent experiments showed that insulin crystallized from a solution containing between 20 and 30 units per cc. yielded larger crystals with sharper edges, provided once crystallization started ·the liquid was undisturbed and allowed to cool very slowly. Slow cooling may be accomplished by immersing the flask or beaker in a larger container of warm water at about 35–40°.

The following determinations were made.

Melting Point—The method given in the United States Pharmacopoeia was followed for the determination of the melting point of the crystals.

TABLE I

Results of Determinations on Crystalline Insulin

	Per cent	Averages	Spectrographic determinations	Per cent
		per cent		
Ash	0 52	0.55	Zinc	0.153
	0 58		Cobalt	0.0001
Zinc	0.142	0.149	Nickel	0.0004
	0 155		Cadmium	0.001
Nitrogen	15.4	15· 5		
	15.6			
Sulfur	2.96	2.96		
Moisture	3 0	3.0		

Potency, 22 to 23 i.u. per mg.

Ash—Ashing was performed in platinum crucibles by first charring the crystals over a free flame and then transferring the crucibles to an electric oven regulated to a maximum temperature of about 600°.

Zinc—The zinc determination was performed according to the method of Sahyun and Feldkamp (4).

Nitrogen—Nitrogen was determined by the usual standard Kjeldahl method.

Sulfur—Microdetermination of sulfur was carried out through the kindness of Dr. E. W. Schoeffel.

Spectrographic Analysis—Spectrographic analyses for zinc, cobalt, nickel, and cadmium were kindly performed by the X-ray and Spectrography Department of The Dow Chemical Company.

Potency—The rabbit method of assay, according to the procedure of Hershey and Lacey (5), was employed for the determination of the potency of this preparation.

A summary of the results is given in Table I.

SUMMARY

A crystalline insulin containing as little zinc as 0.15 per cent, cobalt 0.0001 per cent, nickel 0.0004 per cent, and cadmium 0.001 per cent, and the method of its preparation are herein described. It assayed about 22 i.u. per mg.

BIBLIOGRAPHY

1. Cohn, E. J., Ferry, J. D., Livingood, J. J., and Blanchard, M. H., *Science*, **90**, 185 (1939).
2. Scott, D. A., *Biochem. J.*, **28**, 1592 (1934).
3. Maxwell, L. C., and Feldkamp, R. F., read before the Division of Biological Chemistry, American Chemical Society, Detroit, September 12 (1940).
4. Sahyun, M., and Feldkamp, R. F., *J. Biol. Chem.*, **116**, 555 (1936).
5. Hershey, J. M., and Lacey, A. H., *League of Nations, Quart. Bull. Health Organisation, Biol. Standardisation II*, 595 (1936).

THE MUCOPOLYSACCHARIDES OF SKIN*

By KARL MEYER AND ELEANOR CHAFFEE

(From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, and the Institute of Ophthalmology, Presbyterian Hospital, New York)

Intracutaneous injection of some extracts of animal and bacterial origin, together with a suitable indicator, causes the spreading or diffusion of the indicator over a wide area. This phenomenon, which has been called the "spreading" or "diffusion" reaction (1, 2), is of interest in the study of bacterial invasion and possibly in the problem of capillary permeability. It has been established recently that a close relationship exists between most "spreading" factors and an enzyme hydrolyzing the mucopolysaccharide, hyaluronic acid (3, 4), found several years ago in our laboratory (5, 6). It was shown by a number of investigators that, with a few notable exceptions, extracts containing "spreading" factors likewise contained the enzyme hyaluronidase. It was therefore desirable to establish the presence of the substrate of this enzyme in skin.

The presence of a mucin-like material in the skin has been established previously both by chemical and histological methods.[1] Van Lier in 1909 (8) obtained a viscous "mucin" containing sulfuric acid from the skin of different animals. An apparently similar material was obtained by Claude, who further showed that it was hydrolyzed by leech extracts (9). Bensley (10) and Sylvén (11) demonstrated a mucin-like interfibrillar substance in skin by histological methods.

[1] In beef skin lactose was demonstrated by the orcinol method of Sørensen (7). It has been previously pointed out that this method fails to distinguish between galactose and glucuronic acid. Glucuronic acid obviously was not included in the standards.

The work of van Lier and our own study on the mucopolysaccharide of cornea (12) had led us to suspect a sulfuric acid-containing polysaccharide in skin similar to the cornea polysaccharide, since these two tissues are closely related embryologically. Our first polysaccharide fractions from skin indeed contained sulfuric acid and the material was hydrolyzed as was the cornea polysaccharide by various hyaluronidases, though the rate of hydrolysis differed markedly from that of the cornea fraction. Furthermore, while the analytical data indicated equimolar concentrations of hexosamine, hexuronic acid, and acetyl, the figures for sulfur gave varying equivalents. It then became obvious that we were dealing with mixtures of different mucopolysaccharides, one sulfate-free and the other containing sulfate, but both composed of equimolar concentrations of hexosamine, hexuronic acid, and acetyl.

The separation of two fractions was made possible by two methods: (1) On complete hydrolysis of the mixture by pneumococcus enzyme, the sulfate-containing polysaccharide was left in solution. (2) On fractionation by alcohol in the presence of an excess of barium acetate, a sharp separation of the two polysaccharides could be obtained. By chemical and enzymatic analysis the sulfate-free mucopolysaccharide could be shown to be identical with hyaluronic acid. From sulfate-containing fractions, chondrosamine was obtained as the only hexosamine present, thus indicating chondroitinsulfuric acid. However, the rotation of three preparations was considerably higher than that of chondroitinsulfuric acid prepared from cartilage (13) or umbilical cord. In one preparation, however, the rotation was identical with that of chondroitinsulfuric acid. The most probable explanation of these findings seems to be that there exists in skin, beside chondroitinsulfuric acid, an as yet unknown isomer of this acid of higher specific rotation.

EXPERIMENTAL

The starting material was fresh pig skin. In the first experiments 50 per cent urea solution brought to pH 9 with sodium hydroxide was used as an extractant. By this method good preparations of the mixture of the carbohydrates were obtained, though the yield was small and the time necessary for the extrac-

tion was too long. Extraction with alkali was therefore employed. The following example illustrates the procedure used.

The inside of the skin was cleaned of fat as far as possible with a sharp knife, and the skin ground. 2.28 kilos were then incubated with 6 liters of normal sodium hydroxide at 37° for 28 hours. The almost completely liquefied material was neutralized with 50 per cent acetic acid and filtered with the aid of Hyflo Super-cel. The solution was poured into 2 volumes of alcohol and centrifuged after standing overnight. The precipitate was washed with alcohol, taken up in 500 cc. of 10 per cent calcium chloride made just alkaline to phenolphthalein, and shaken with 100 cc. of a mixture of 4 parts of chloroform and 1 part of amyl alcohol. The suspension was centrifuged, the aqueous supernatant solution again filtered through Filter Cel and poured into 2 volumes of alcohol, and the precipitate washed with alcohol and dissolved in 200 cc. of water. 8 gm. of Lloyd's reagent and sufficient glacial acetic acid to give a 6 per cent acetic acid solution (1 N) were added. The mixture was centrifuged, the residue washed once with normal acetic acid, and the combined solutions poured into 2 volumes of alcohol. The precipitate was washed with alcohol and ether and dried. The residue was dissolved in 100 cc. of 1 per cent sodium acetate solution, brought to a pH of about 6 with normal acetic acid, and incubated for about 6 hours with 2 cc. of filtered saliva in the presence of toluene, in order to digest glycogen, still present at this stage. The solution was again poured into 2 volumes of alcohol, and the precipitate washed with alcohol and extracted with 100 cc. of barium acetate solution. Alcohol was added to the extract to give a 20 per cent concentration. The precipitate formed after standing in the cold overnight was removed by centrifuging. This precipitate was the sulfuric acid-containing fraction. It was washed, extracted with small portions of water, and poured into 5 volumes of glacial acetic acid. The precipitate was removed immediately, washed with alcohol, acetone, and ether, and dried; yield 1.85 gm.

The clear, supernatant 20 per cent alcohol solution contained hyaluronic acid, which was precipitated by bringing the alcohol concentration to 77 per cent. The stringy precipitate was removed by centrifuging, washed with alcohol, and extracted with small portions of water. The combined solutions were poured

into 5 volumes of glacial acetic acid. The precipitate was removed immediately, washed, and dried; yield 1.08 gm. In some preparations the hyaluronic acid fraction was further purified by zinc acetate precipitation followed by a second precipitation from acetic acid as described formerly (14).

In the purification of the sulfuric acid fraction, digestion with pneumococcus hyaluronidase was used in all preparations, in order to remove possible traces of hyaluronic acid. The digestion was ordinarily carried out directly after the precipitation as the barium salt. For this purpose the precipitate was dried *in vacuo* to remove alcohol and dissolved in water. The pH was adjusted to 5.8, 5 mg. of enzyme were added, and the mixture incubated in the presence of toluene for 2 days. The enzyme was removed by adsorption on Lloyd's reagent in normal acetic acid, and the

TABLE I

Analysis of Hyaluronic Acid Fractions

Preparation No.	Nitrogen	Hexosamine	Uronic acid	Acetyl	Equivalent weight	$[\alpha]_D$ in neutral solution	Equivalents per equivalent weight			
							Nitrogen	Hexosamine	Uronic acid	Acetyl
	per cent	per cent	per cent	per cent		degrees				
I	2.99	39.7	41.9	13.1	475	−74.9	1.02	1 05	1.03	1.45
II	2.78	37.2	42.0	9 50	455	−67.5	0 91	0.95	0.98	1 01

polysaccharide precipitated first from alcohol and then from 5 volumes of glacial acetic acid. In each case the finished product was retested for hyaluronic acid by the pneumococcus enzyme by our routine procedure with a 1 per cent solution (6). The hydrolysis was negative in all samples.

In Table I the analytical data of two hyaluronic acid preparations from skin are given. The analytical figures and the rotation are typical for hyaluronic acid.

In Fig. 1 the hydrolysis of hyaluronic acid from skin and from umbilical cord is compared. It is obvious from the figure that the kinetics of the hydrolysis are identical in both polysaccharides.

In Table II the analytical data of the sulfuric acid fractions are summarized. It can be seen from Table II that the substance is composed of equimolar amounts of hexosamine, hexuronic acid,
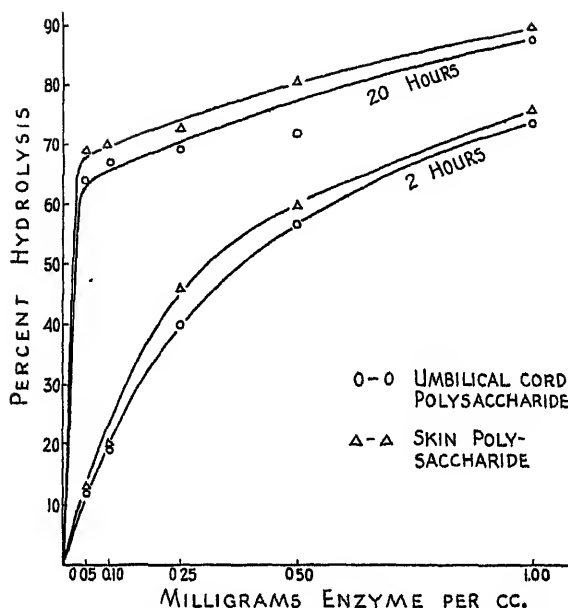
Fig. 1. 1 per cent solutions of hyaluronic acid prepared from umbilical cord and the sulfur-free polysaccharide prepared from skin were incubated with varying amounts of pneumococcus enzyme at 37°, pH 5.8, in the presence of toluene. Reducing values were determined by the ceric sulfate method, and the per cent hydrolysis calculated from the glucose equivalents of glucuronic acid and acetylglucosamine, calculated from glucosamine, determined by analysis.

TABLE II

Analysis of Sulfur-Containing Fractions

Prep-ara-tion No.	Nitro-gen	Hexos-amine	Uron-ic acid	Acetyl	Sul-fur*	Equivalent weight	$[\alpha]_D$ in neutral solu-tion	Equivalents per equivalent weight				
								Nitro-gen	Hexos-amine	Uron-ic acid	Acetyl	Sul-fur
	per cent	*per cent*	*per cent*	*per cent*	*per cent*		*degrees*					
I	2.24	27 0	32 5	8 33	5 98	547	−53.9	0.88	0.85	0.91	1.06	1.02
II	2 15	27 3	32.5	6.10	5.12	668	−28 4	1.03	1.02	1.12	0.95	1.10
III	2 19	30.5	32.5	8 95	5.22	525	−57.9	0 82	0.89	0 88	1.09	0.86
IV	2 05	25.1	30 3	7 84	5.07	620	−55.1	0.91	0.87	0.96	1 13	0.98

* We thank Mr. Henry S. Bloch for these microdeterminations.

acetyl, and sulfuric acid.   It seems remarkable that the rotation of three preparations varied between $-54°$ and $-58°$, while Preparation II had a rotation of $-28.4°$.   Chondroitinsulfuric acid prepared from umbilical cord by exactly the same method as that used in skin gave an $[\alpha]_D$ of $-16.4°$.   All rotations were determined on the weighed samples neutralized with the calculated amount of sodium hydroxide.   All samples gave a strong uronic acid reaction with naphthoresorcinol.

The hexosamine was isolated from a mixture of Preparations II and III of Table II (430 mg. of Preparation II, 317 mg. of Preparation III), according to the method published previously (14). 85 mg. of a hexosamine hydrochloride were obtained.   This was

TABLE III

Analysis of Crystalline Hexosamine Hydrochloride

| | N (Dumas)* | Cl | $[\alpha]_D$ | |
			Initial	Final
	per cent	per cent	degrees	degrees
Found	6.28	16 8	+72 6	+94.2
Chondrosamine HCl, theoretical	6 49	16.4		+95 (Levene (15))

* We thank Mr. Henry S. Bloch for the microdeterminations.

recrystallized once.   The results presented in Table III show that the substance was chondrosamine hydrochloride.

The hexosamine was also isolated from a fraction with a high rotation (Preparation IV).   From 0.984 gm., 88.5 mg. were isolated in a first fraction, which, however, contained inorganic material.   The N found was 5.70 per cent, $[\alpha]_D$ at equilibrium $+87.0°$.   Corrected for the ash, the $[\alpha]_D$ becomes $+98.0°$.   The mutarotation was upward.   The second fraction was 37.5 mg. The N found was 6.22 per cent, Cl 16.2 per cent, $[\alpha]_D$ at equilibrium $+93.6°$ (mutarotation upward).   Thus this fraction was likewise chondrosamine hydrochloride.   A third fraction was obtained on prolonged standing at $0°$, weighing 13 mg.   The N (Kjeldahl) was 6.0 per cent (the Kjeldahl values on hexosamine are always too low), Cl 16.0 per cent, $[\alpha]_D$ at equilibrium $+94.3°$ (mutarotation upward).   Thus this fraction likewise contained only chondrosamine.

It has to be stressed that from mixtures of chondrosamine and glucosamine hydrochlorides, the latter is always obtained first on account of its greater insolubility. The rotation of the hexosamine isolated from mixtures of chondroitin- and mucoitinsulfuric acids therefore is lower in the first fractions than that of pure chondrosamine (14).

In contrast to the pneumococcus enzyme preparations, enzyme prepared from bovine testis slowly attacked the sulfuric acid fraction of skin with simultaneous liberation of sulfate. The rate of hydrolysis of Preparation IV was much slower than that of a preparation of the barium salt of chondroitinsulfuric acid from cartilage.

DISCUSSION

The presence of hyaluronic acid in skin has been established, thus furnishing a further argument in favor of the identity of the "spreading" factor and hyaluronidase. Furthermore, since the enzyme hyaluronidase has been demonstrated in skin in rather high concentration (16), we have concluded that the skin in analogy with other enzyme substrate systems is probably an organ of great activity in hyaluronic acid metabolism.

The nature of the sulfuric acid fraction is at the present time still uncertain. Until recently only three hexosamine- and sulfuric acid-containing systems have been known, two containing glucosamine, namely mucoitinsulfuric acid and heparin, and one containing chondrosamine, namely chondroitinsulfuric acid. However, proof was furnished by this laboratory that at least two groups of mucoitinsulfuric acids could be distinguished, one isolated from cornea and one from gastric mucosa (12). Both evidently were dissimilar in composition and enzymatic hydrolysis to heparin. The cornea polysaccharide is the monosulfuric acid ester of hyaluronic acid, whereas the gastric mucoitinsulfuric acid is still unknown in its composition, except that it contains glucosamine, acetyl, uronic acid, and sulfuric acid in equimolar concentrations. It further occurs partly as a disulfuric acid (17).

A search in the literature for differences in properties of chondroitinsulfuric acid from different sources and species proved unsuccessful. No rotations have been reported for chondroitinsulfuric acid prepared from the following sources: bovine cartilage,

tendons, aorta, and sclera (15), nasal septum of pigs, cartilage of shark and ray (18), nasal septum of whale (19). The only rotation of chondroitinsulfuric acid in the literature is that given by Jorpes (20) who found an $[\alpha]_D$ of $-17.4°$. This rotation is obviously that of the acid salt. It is identical with the values of the acid salt previously reported from our laboratory ($-17.4°$, $-17.6°$, $-15.9°$)[2] (13). The source of Jorpes' as well as of our material was bovine tracheal cartilage. The difference found between the rotation of this chondroitinsulfuric acid ($-30°$) and that found in the skin polysaccharide ($-56°$) does not seem to be explained by partial hydrolysis of the latter, since hydrolysis of chondroitinsulfuric acid leads to dextrorotation. Furthermore the rotation of a sample of chondroitinsulfuric acid prepared from human umbilical cord by exactly the method used in the pig skin material gave a polysaccharide with $[\alpha]_D$ of $-16.4°$. It also does not seem probable that an impurity causes the high rotation. After reprecipitation from alcohol and acetic acid, the rotation remained unchanged. A sample analyzed electrophoretically did not show a foreign component. It migrated as a broad boundary in 0.025 M barbiturate buffer at pH 7.6 with an average mobility of about -14×10^{-5} cm.[2] volt^{-1} sec.$^{-1}$ (Dr. D. H. Moore).

At the present time it cannot be decided whether species or organ differences exist between chondroitinsulfuric acids of different origin or whether we are always dealing with isomeric mixtures, occurring in different ratios. In another mucopolysaccharide, hyaluronic acid, no essential difference was found in samples prepared from vitreous humor of cow or pig (21), or from a human tumor (22) or bovine synovial fluid (23). However, in heparin a remarkable difference in activity was recently found in the crystalline barium salts prepared from different species (24). Obviously chemical differences must correspond to these physiological differences. From the analytical data it previously appeared doubtful whether heparin was a pure and single substance (17).

SUMMARY

A mucopolysaccharide mixture was obtained from pig skin. This mixture was separated by enzymatic hydrolysis and by

[2] The $[\alpha]_D$ on neutralization changes to a value of $-30°$. This is probably due to the opening of the lactone ring of the glucuronic acid part of the molecule.

fractionation with alcohol in the presence of barium into two components. One was shown to be identical with hyaluronic acid by analysis and enzymatic hydrolysis. The occurrence of hyaluronic acid in skin is taken as an additional indication for the identity of the "spreading" factor and hyaluronidase. The second component contained hexosamine, acetyl, a uronic acid, and sulfuric acid in equimolar ratios. The hexosamine proved by rotation to be chondrosamine. The rotation of three out of four samples, however, was considerably higher than that of chondroitinsulfuric acid prepared from beef cartilage.

BIBLIOGRAPHY

1. Hoffman, D. C., and Duran-Reynals, F., *J. Exp. Med*, **53**, 387 (1931).
2. McClean, D., *J. Path. and Bact.*, **33**, 1045 (1930).
3. Chain, E., and Duthie, E. S., *Nature*, **144**, 977 (1939).
4. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *Proc. Soc. Exp. Biol. and Med.*, **44**, 294 (1940).
5. Meyer, K., Dubos, R., and Smyth, E. M., *J. Biol. Chem.*, **118**, 71 (1937).
6. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, **71**, 137 (1940).
7. Grassmann, W., and Schleich, H., *Biochem. Z*, **277**, 320 (1935). Grassmann, W., Janicki, J., Klenk, L., and Schneider, F., *Biochem. Z.*, **294**, 95 (1937).
8. van Lier, E. H. B., *Z. physiol. Chem.*, **61**, 177 (1909).
9. Claude, A., *Proc. Soc. Exp. Biol. and Med*, **43**, 684 (1940).
10. Bensley, S. H., *Anat. Rec.*, **60**, 93 (1934).
11. Sylvén, B., *Virchows Arch. path. Anat*, **303**, 280 (1938).
12. Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, **43**, 487 (1940); *Am. J. Ophth.*, **23**, 1320 (1940).
13. Meyer, K., and Smyth, E. M., *J. Biol. Chem.*, **119**, 507 (1937).
14. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **114**, 689 (1936).
15. Levene, P. A., Hexosamines and mucoproteins, Monographs on biochemistry, London and New York (1925).
16. Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, **73**, 309 (1941).
17. Meyer, K., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6** (1938).
18. Fürth, O., and Bruno, T., *Biochem. Z.*, **294**, 153 (1937).
19. Miyazaki, T., *J. Biochem.*, Japan, **20**, 223 (1934).
20. Jorpes, E., *Biochem. Z.*, **204**, 354 (1929).
21. Meyer, K, Smyth, E. M., and Gallardo, E., *Am. J. Ophth.*, **21**, 1083 (1938).
22. Meyer, K., and Chaffee, E., *J. Biol. Chem.*, **133**, 83 (1940).
23. Meyer, K., Smyth, E. M., and Dawson, M. H., *J. Biol. Chem.*, **128**, 319 (1939).
24. Jaques, L. B., *Science*, **92**, 489 (1940).

THE EFFECT OF 2,3,5-TRIIODOBENZOATE AND MONO-IODOACETATE ON THE OXIDATION OF CERTAIN SUBSTANCES BY RAT BRAIN

By FREDERICK BERNHEIM AND MARY L. C. BERNHEIM

(From the Departments of Physiology and Pharmacology and Biochemistry, Duke University School of Medicine, Durham, North Carolina)

Monoiodoacetate has been shown to be an inhibitor of a number of reactions in mammalian tissues and in yeast. Recently Barker, Shorr, and Malam (1) showed that in brain it inhibited glycolysis in concentrations which had no effect on the oxidation of glucose. Peters, Rydin, and Thompson (2) found that iodoacetate caused an accumulation of pyruvic acid in pigeon brain. It is the purpose of this paper to present in more detail the effect of monoiodoacetate on the oxidation by brain of glucose and lactic and pyruvic acids and to compare its effects with those of 2,3,5-triiodobenzoate, an organic iodine compound about which nothing has been reported.

EXPERIMENTAL

Four rat brains were chopped with scissors, ground in a mortar, and squeezed through muslin. The resulting suspension was then washed three times with 50 cc. of M/30 phosphate buffer, pH 7.8, and finally made up to 25 cc. with 0.05 M buffer. 1 cc. of the suspension was used in each Warburg vessel. The oxygen uptake of this suspension is 10 per cent or less of the original. Because very little work has been done with washed brain tissue, the rate and extent of the oxygen uptake of glucose and pyruvic acid were compared when these substances were added to the original suspension and to the equivalent amount of material washed three times. Fig. 1 shows that washing increases the rate of oxidation of glucose and pyruvate. The same is true for lactate. Washing with water instead of phosphate results in an inactive preparation.

Fig. 2 shows the concentration curves for the three substrates. Pyruvate gives proportional oxygen uptakes from 0.2 to 2.0 mg. The oxygen uptake of the lactate increases throughout the same range but the proportionality is lost above 0.4 mg. With glucose the proportionality is also lost above 0.4 mg. but there is no further increase in oxygen uptake with increasing amounts. The oxygen uptake of glucose corresponds to 10 atoms, of pyruvate, 2 atoms, and of lactate, 2 to 2.5 atoms of oxygen per molecule. The respective respiratory quotients are 1.0, 1.0, and 0.8.
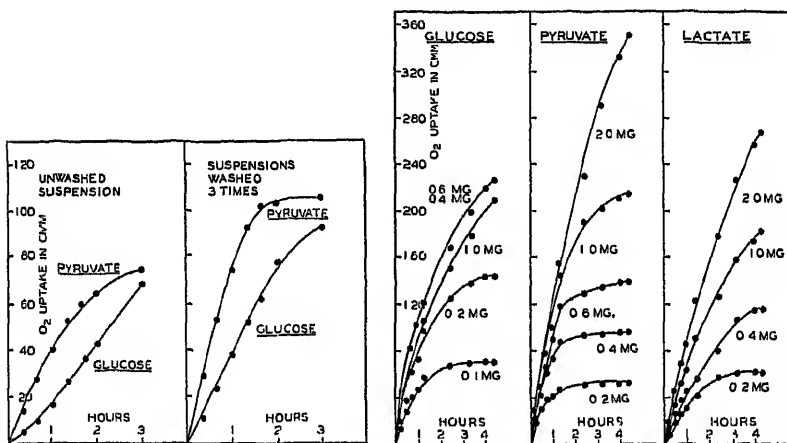
FIG. 1.   FIG. 2.

FIG. 1. The oxidation of 0.5 mg. of pyruvate and 0.25 mg. of glucose by equal amounts of washed and unwashed rat brain suspension at pH 7.8, 37°. The uptake of the controls has been subtracted from the uptake of the brain with substrate.

FIG. 2. The oxidation of different amounts of glucose, pyruvate, and lactate by washed brain suspension at pH 7.8, 37°.

The relative rates of oxygen uptake with equimolar concentrations of the three substrates vary with the hydrogen ion concentration. This is shown in Fig. 3. At pH 7.8 the rate for pyruvate is greater than that for lactate and glucose. At pH 6.7 the rate for pyruvate and lactate is approximately the same but is slower for glucose.

The monoiodoacetic acid was recrystallized from petroleum ether, and the 2,3,5-triiodobenzoic acid from glacial acetic acid. At a concentration of 2.7×10^{-4} M the former causes complete

inhibition of the oxidation of glucose, and 0.5×10^{-4} M causes a 32 per cent inhibition. This concentration is 1.5 times greater
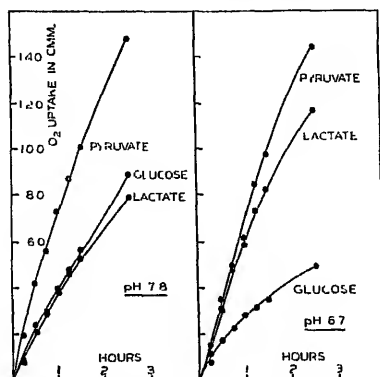
FIG. 3. The oxidation of 1.0 mg. of pyruvate, 2.2 mg of glucose, and 1.0 mg. of lactate by washed brain suspension at pH 7.8 and 6.7, 37°.
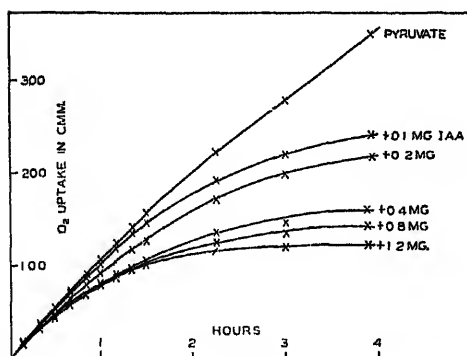
FIG. 4.

FIG. 5.

FIG. 4. The effect of different concentrations of monoiodoacetate (2.7×10^{-4} to 3.2×10^{-3} M) on the oxidation of 2.0 mg. of pyruvate by washed brain suspension at pH 7.8, 37°.
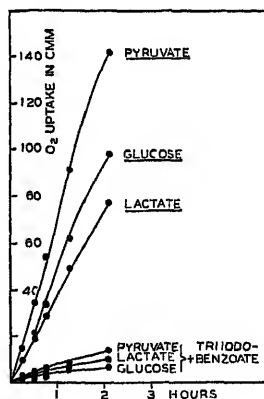
FIG. 5. The effect of 1 0 mg. (1.0×10^{-3} M) of 2,3,5-triiodobenzoate on the oxidation of 1.0 mg. of pyruvate, 1.0 mg. of lactate, and 2.2 mg. of glucose by washed brain suspension at pH 7.8, 37°

than that necessary to inhibit glycolysis completely (1). The effect of monoiodoacetate on pyruvate oxidation is shown in Fig. 4. Depending on the concentration, inhibition occurs from

15 to 90 minutes after the addition of the drug. This is true whether it is added with the pyruvate or 15 to 90 minutes before it. In other words, monoiodoacetate causes a gradual inactivation of the pyruvate enzyme in contrast to its immediate action on the glucose system. On the lactate oxidation its action is similar to that on pyruvate presumably because some lactate is oxidized to pyruvate.

On the other hand triiodobenzoate causes an immediate inhibition of all three oxidations. This is shown in Fig. 5. A concentration of 1.0×10^{-3} M causes an 80 to 90 per cent inhibition. The inhibition decreases with decreasing concentrations of the drug and disappears at 3.0×10^{-4} M.

The inhibition by the drugs on the oxidation of the three substrates is not affected by a change of pH from 7.8 to 6.7 nor by the addition of thiamine or fumarate. The concentration of substrate is also without effect.

DISCUSSION

Triiodobenzoate has very little effect on the oxygen uptake of liver and kidney. It inhibits the oxygen uptake of unwashed brain suspension 45 to 55 per cent. In the brain it inhibits the systems that are also inhibited by monoiodoacetate. The latter is much more effective in inhibiting the oxidation of glucose. The reverse is true for the oxidation of lactate and pyruvate. Neither compound in the concentrations used has any effect on the oxidation of succinate which, according to Quastel (3), is the only other substrate adequately oxidized by brain *in vitro*. 3,5-Diiodo-2-hydroxybenzoate acts as triiodobenzoate does. On the other hand, 3,5-diiodo-4-hydroxybenzoate in the same concentration has no effect on the pyruvate oxidation and only a slight effect on the glucose and lactate oxidation.

SUMMARY

1. The washing of brain suspensions with phosphate buffer until the residual oxygen uptake is 10 per cent or less of the original increases the rate of oxidation of added pyruvate, lactate, and glucose. Concentration curves for the three substrates are given.

2. 2.7×10^{-4} M monoiodoacetate immediately and completely

inhibits the oxidation of glucose. The oxidation of pyruvate and lactate is inhibited by this concentration only after a 90 minute incubation. Higher concentrations require a shorter incubation period.

3. 1.0×10^{-3} M 2,3,5-triiodobenzoate immediately inhibits the oxidation of the three substrates. The inhibition is 80 to 90 per cent, decreases with decreasing concentrations, and is absent at 3.0×10^{-4} M.

BIBLIOGRAPHY

1. Barker, S B., Shorr, E., and Malam, M., *J. Biol. Chem*, **129**, 33 (1939).
2 Peters, R. A., Rydin, H., and Thompson, R. H. S., *Biochem. J.*, **29**, 63 (1935).
3. Quastel, J. H., *Physiol. Rev.*, **19**, 135 (1939).

INHIBITION OF THE d-AMINO ACID OXIDASE BY BENZOIC ACID

By J. RAYMOND KLEIN and HENRY KAMIN

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

Benzoic acid has been reported to inhibit the oxidation of succinic acid by muscle (1), the oxidation of butyric acid and crotonic acid by liver slices (2), and the oxidation of acetoacetic acid by kidney slices (3). It has also been reported to inhibit the oxygen uptake of liver and kidney slices, diaphragm, minced liver, kidney, and brain (4).

In the present work it was found that benzoic acid had a slight inhibitory effect on the oxygen uptake of broken cell preparations of rat liver and kidney. It had no effect on the oxidation of succinic acid, lactic acid, hypoxanthine, xanthine, uric acid, choline, tyramine, $l(-)$-tyrosine, $l(-)$-proline, $l(-)$-phenylalanine, sarcosine, or ethyl alcohol by the liver preparation. The oxidation of d-amino acids by various preparations of the d-amino acid oxidase was markedly inhibited by benzoic acid.

EXPERIMENTAL

Effect of Benzoic Acid on Oxidation of d-Amino Acids—The oxidation of d-amino acids by rat liver and kidney slices and by the preparations of the d-amino acid oxidase described below and the effect of benzoic acid on the oxidation were tested by measuring oxygen uptakes manometrically. The measurements were made with the usual Warburg apparatus at 37.5°.

The extra oxygen uptake of liver slices in the presence of 0.0075 M $d(-)$-alanine was 30 microliters per 30 minutes per 100 mg. of wet tissue. This extra oxygen uptake was completely inhibited by 0.001 M benzoic acid. The presence of glycine did not affect the inhibitory effect of benzoic acid. The benzoic acid did not

inhibit the oxygen uptake of the liver slices in the absence of the amino acid. The extra oxygen uptake of kidney slices in the presence of 0.0075 M *d*(−)-alanine was 53 microliters per 30 minutes per 100 mg. of wet tissue. 0.001 M benzoic acid inhibited the rate 50 per cent. The oxygen uptake of the kidney slices in the absence of the substrate was inhibited 6 per cent by benzoic acid.

The oxidation of 1 mg. of *d*(−)-alanine, *d*(+)-phenylalanine, and the *d* isomer of *dl*-proline by broken cell preparations (5) of rat liver and kidney and the washed proteins (6) of rat liver was completely inhibited by 0.01 M benzoic acid at pH 7.0, 7.5, and 8.0. With 0.001 M benzoic acid the rate of oxidation of these amino acids was inhibited about 90 per cent. In most cases the oxygen uptake of the broken cell preparations alone was inhibited about 10 per cent at pH 6.6 and 5 per cent at pH 8.1. The inhibition usually decreased as the pH approached 7.4, and then increased up to pH 8.1. In a few experiments the inhibition was negligible at pH 6.6 to 8.1.

Benzoic acid inhibited the activity of two purified preparations of the *d*-amino acid oxidase. One preparation was made by extracting 1 gm. of dry pig kidney (7) with 10 ml. of water, and then centrifuging. 1 ml. of the supernatant liquid plus 1 ml. of 0.1 M pyrophosphate buffer, pH 8.3, was used to oxidize the amino acids. The other preparation was a reconstructed oxidase composed of 3 to 6 mg. of a preparation of the protein fraction of the oxidase plus 2.4 to 4.8 γ of flavin-adenine dinucleotide. The protein preparation was made from the dry pig kidney and the flavin from yeast (7). Neither preparation of the oxidase could oxidize *l*-amino acids.

Data illustrating the effect of benzoic acid on the oxidation of *d*-amino acids by the reconstructed oxidase are given in Fig. 1. The data show that 10^{-4} M benzoic acid inhibits the activity 79, 52, and 50 per cent in the case of alanine, methionine, and proline respectively. The extent of inhibition is calculated from the oxygen uptakes at 10 minutes. In addition, the rate of oxidation of 1 mg. of *d*(+)-phenylalanine, *d*(+)-leucine, and *d*(+)-tyrosine was found to be inhibited 57, 28, and 64 per cent by 10^{-4} M benzoic acid. The data for methionine in Fig. 1 show that the extent of oxidation was not affected by benzoic acid. This was

also true in the case of the other amino acids when the experiments were of longer duration. Similar data were obtained with the kidney extract. The benzoic acid also inhibits the rate of reduction of methylene blue by mixtures of the oxidase preparations and amino acids.

It can be shown from the data in Fig. 1 that the oxidation of the amino acids in the presence and absence of benzoic acid is a first order reaction; *i.e.*, a straight line is obtained when the logarithm of the concentration of unoxidized amino acid is plotted as a function of time. This indicates that the enzyme is not pro-
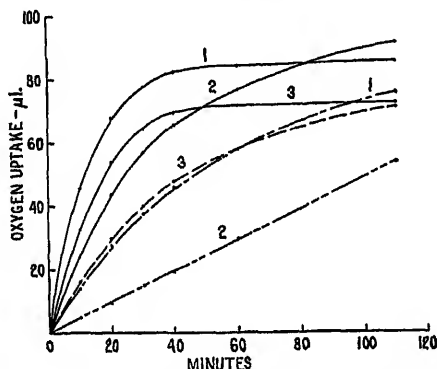
FIG. 1. Effect of benzoic acid on the activity of a reconstructed *d*-amino acid oxidase. The continuous lines represent oxidation of amino acids in the absence of benzoic acid, broken lines, oxidation in the presence of 10^{-4} M benzoic acid. The oxidase consisted of 3 ml. of the protein preparation and 2.4 γ of flavin-adenine dinucleotide in 2 ml. of 0.05 M pyrophosphate buffer, pH 8.3. Curves 1, 0.0038 M *dl*-proline; Curves 2, 0 0056 M *dl*-alanine, Curves 3, 0 0034 M *dl*-methionine.

gressively inhibited by benzoic acid. This is also indicated by the fact that incubation of the enzyme and benzoic acid for 30 minutes before the addition of the amino acid does not affect the extent of inhibition.

A comparison of the effect of benzoic acid and that of other active acids is given in Table I. Under the same conditions isophthalic, hippuric, nicotinic, quinoline-β-carboxylic, and pyrazinecarboxylic acids were slightly active (0.08 or less), while *p*-aminobenzoic, phthalic, phenylacetic, picolinic, quinolinic, dinicotinic, 6-hydroxynicotinic, pyrimidine-4-carboxylic, acetic,

iodoacetic, stearic, and malonic acids, and benzamide, aniline, phenol, toluene, benzene, nicotinamide, trigonelline, pyridine, and glycine were inactive. These data show that compounds containing the benzene ring are more effective than those containing other rings, that the carboxyl group is necessary for inhibition, and that further substitution in the ring, or between the ring and the carboxyl group, depresses the effectiveness of the compound.

The following observations indicate that the inhibition produced by benzoic acid is reversible. (*a*) The inhibitory effect disappeared after dialysis. (*b*) Preparations of the protein

TABLE I

Comparison of Inhibitory Effect of Benzoic Acid on d-Amino Acid Oxidase with Effect of Other Compounds

The activity of the kidney extract was tested in the presence of 2 mg. of *dl*-alanine. The decrease in activity produced by each substance was calculated from the oxygen uptakes at 10 minutes. The measure of inhibition given in the table is the ratio of the decrease in activity produced by the substance in 10^{-4} M concentration to that produced by 10^{-4} M benzoic acid. This concentration of benzoic acid decreased the activity 60 per cent.

Substance	Inhibition, 10^{-4} M benzoic acid = 1
o-Aminobenzoic acid	0.25
m-Aminobenzoic "	0.33
o-Hydroxybenzoic acid	0.32
m-Hydroxybenzoic "	0.21
Terephthalic acid	0.19
2,4,5-Triiodobenzoic acid	0.13
p-Nitrobenzoic acid	0.80

component of the oxidase made from kidney extract to which benzoic acid was added had the same activity as preparations made from untreated extracts. (*c*) The protein component can be recovered without loss of activity from solutions containing benzoic acid by treatment (7) with acid and ammonium sulfate. (*d*) Flavin-dinucleotide precipitated as the silver salt (7) from solutions containing benzoic acid had the same activity, after regeneration with hydrogen sulfide, as that precipitated from pure aqueous solution.

Assuming the formation of a reversibly dissociating substrate-enzyme complex and inhibitor-enzyme complex as suggested by

Michaelis and Menten (8), the relation between the concentrations of the components of an oxidase solution containing amino acid and benzoic acid would be

$$\text{(I)} \qquad \frac{(\text{Amino acid}) \, (\text{benzoic acid-oxidase})}{(\text{Benzoic acid}) \, (\text{amino acid-oxidase})} = \frac{K_{\text{amino acid}}}{K_{\text{benzoic acid}}}$$

The parentheses indicate concentration and K the dissociation constants of the complexes.

TABLE II

Calculation of $K_{\text{amino acid}}/K_{\text{benzoic acid}}$ from Equation I

The reconstructed oxidase was used in Experiments 1, 2, 3, and 4. 3 mg. of protein preparation plus 2.4 γ of flavin-dinucleotide were used in Experiments 1 and 4, 3 mg. of protein plus 4.8 γ of flavin in Experiment 3, and 6 mg. of protein plus 2.4 γ of flavin in Experiment 2. The kidney extract was used in the other experiments. The oxygen uptakes given in Experiments 2, 3, and 4 are for 7.5 minutes; the others for 10 minutes.

Experiment No.	Concentration of $d(-)$-alanine	Concentration of benzoic acid	Oxygen uptake			$\dfrac{K_{\text{amino acid}}}{K_{\text{benzoic acid}}}$
			Without benzoic acid	With benzoic acid	Inhibition	
	M	M	*microliters*	*microliters*	*microliters*	
1	0 0028	0 0001	24	5	19	106
2	0 0056	0.0001	72	24	48	112
3	0 0056	0 0001	48	16	32	112
4	0.0056	0.0001	44	14	30	120
5	0.0560	0 0001	45	36	9	140
6	0 0280	0 0001	35	24	11	128
7	0 0056	0 0001	20	8	12	84
8	0 0028	0.00001	21	14	7	140
9	0.0028	0 0001	21	5	16	90
10	0 0028	0.001	21	0.5	20 5	115
Mean.	.	. . .	. . .	. . .		115

In Table II are given data which permit the calculation of the ratio indicated in Equation I and the values of the ratio obtained. The rate of oxygen uptake in the absence of benzoic acid was taken as a measure of the concentration of total oxidase. The rate in the presence of benzoic acid was taken as a measure of (amino acid-oxidase). The difference between these rates was used as a measure of (benzoic acid-oxidase). It was assumed that the concentration of bound amino acid and benzoic acid was small com-

pared to the total concentrations, and that the concentration of amino acid was the same in the absence and presence of benzoic acid. For convenience the initial concentration of *d*-amino acid was used.

Considering the variety of experimental conditions under which the data were obtained, the values for the ratio are in good agreement. Although the agreement cannot be considered as definite proof of the formation of a benzoic acid-oxidase complex, it does make plausible the assumption that the inhibitory effect is due to the formation of such a complex.

The light absorption of solutions of the reconstructed oxidase and of flavin-dinucleotide at wave-lengths of 350 to 500 mμ was not affected by the presence of 10^{-3} M benzoic acid. This suggests that benzoic acid does not combine with the flavin or protein component of the oxidase.

SUMMARY

1. The activity of the *d*-amino acid oxidase was decreased by benzoic acid; *e.g.*, the rate of oxidation of 1 mg. of $d(-)$-alanine by preparations of the oxidase was decreased about 60 per cent by 10^{-4} M benzoic acid.

2. Data were obtained which suggest that the inhibitory effect of benzoic acid may be due to the formation of a *benzoic acid-oxidase* complex.

BIBLIOGRAPHY

1. Mahlen, S., *Skand. Arch. Physiol.*, **53**, 152 (1928).
2. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2143, 2159 (1935).
3. Quastel, J. H., and Wheatley, A. H. M , *Biochem. J.*, **29**, 2773 (1935).
4. Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, **37**, 279 (1037).
5. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **106**, 79 (1934).
6. Bernheim, F., and Bernheim, M. L. C., *J Biol. Chem.*, **127**, 695 (1939).
7. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150, 368 (1938).
8. Michaelis, L., and Menten, M. L., *Biochem. Z* , **49**, 333 (1913).

STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

V. IDENTIFICATION AND SYNTHESIS OF THE HEMORRHAGIC AGENT[*]

By MARK ARNOLD STAHMANN, CHARLES FERDINAND HUEBNER, AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison)

In this communication it is shown through degradation reactions which lead to the chemical synthesis that the hemorrhagic agent, $C_{19}H_{12}O_6$, m.p. 288–289°, isolated by our colleague, Dr. H. A. Campbell, from spoiled sweet clover hay is 3,3'-methylene-bis(4-hydroxycoumarin), formula (I). Before it was necessary for Dr. Campbell to withdraw from the work due to a change in post, it was concluded that the hemorrhagic agent was not identical with any of the 60 or more naturally occurring coumarins previously reported, and that the literature did not contain an account of a substance melting in the range 280–290° whose chemical properties and analysis could be reconciled with those exhibited by the pure hemorrhagic agent (1, 2).

From a careful study of the chemical and physical properties of the crystalline hemorrhagic agent, it became possible to develop a materially shortened extraction procedure[1] which enabled us to accumulate, by repeated mass isolations in the relatively short

[1] Inoculation of the atmosphere in the laboratory and the vessels used in the extraction work with the crystals of the hemorrhagic agent probably facilitated the crystallization (3)

period of 4 months, a large stock of the pure substance (1800 mg.). Work on the elucidation of the structure of this unique substance was then undertaken.

The acidic nature of the hemorrhagic agent has been pointed out previously (1, 2). The red color produced with ferric chloride in cyclohexanone, the stability of the dimethyl ether, $C_{19}H_{10}O_4(OCH_3)_2$, toward alkali, and the formation of a diacetate, $C_{19}H_{10}O_6(OCCH_3)_2$, melting point 250–252° with decomposition, suggested that the acidity was due to two enol structures, rather than to free carboxyl groups. The ease with which the compound dissolves in alkali and the nature of the electrometric titration curve (pK 7.2) indicated that the acidity was not due to phenolic hydroxyls or lactone structures. The test for methoxyl and ethoxyl groups was negative. The presence of carbonyl groups was shown by reactions with phenylhydrazine and hydroxylamine.

Insight into the final structure emerged from the degradation experiments that follow. Consideration will be restricted to the crucial reactions having a direct bearing on the structural diagnosis.

Fusion with potassium hydroxide revealed that 14 of the 19 carbon atoms could be recovered as salicylic acid, $C_7H_6O_3$ (III). The yield of salicylic acid proved to be quantitative in view of the final structure deduced for the parent substance (7.3 mg. of the hemorrhagic agent gave 6.1 mg. of salicylic acid). 5 carbon atoms were eliminated by this treatment. Control fusions with 10 mg. quantities of coumarin gave theoretical yields of salicylic acid.

Degradation in 30 per cent alcoholic potassium hydroxide gave some salicylic acid and a new δ-diketone, $C_{17}H_{16}O_4$ (II), m.p. 101–102°, molecular weight 284. The characterization of this δ-diketone as 1,3-disalicylylpropane by two independent synthetic approaches is given in Paper VI of this series (4).

Degradation in 10 per cent sodium hydroxide gave the δ-diketone quantitatively (11.5 mg. of the hemorrhagic agent yielded 9.5 mg. of the δ-diketone). 2 carbon atoms and 2 oxygen atoms were eliminated by this milder alkali treatment.

When viewed collectively, the products isolated from the alkaline degradations indicated that there are present in the hemorrhagic agent, $C_{19}H_{12}O_6$, two benzene nuclei joined through 5 carbon atoms, the carbon bridge of the δ-diketone; that a car-

bonyl oxygen is located on each carbon atom of the bridge adjacent to the benzene rings; and that ortho to the points of attachment is an oxygen atom which is present as a phenol ester. The formation of salicylic acid from the hemorrhagic agent by more drastic alkaline hydrolysis could be rationalized as involving cleavage of the double bonds of the enol form of the intermediate δ-diketone. These products did not establish the position of the 2 carbon and 2 oxygen atoms eliminated in the formation of the δ-diketone.

Degradation of 3,3'-Methylenebis(4-Hydroxycoumarin) under Alkaline Conditions

I. $C_{19}H_{12}O_6$, 3,3'-methylenebis-(2,4-diketochroman) or 3,3'-methylenebis(4-hydroxy-coumarin)

II $C_{17}H_{16}O_4$, 1,3-disalicylyl-propane

IV. $C_{16}H_{11}O_2N$, anil of 2,4-diketo-chroman

III. $C_7H_6O_3$, salicylic acid
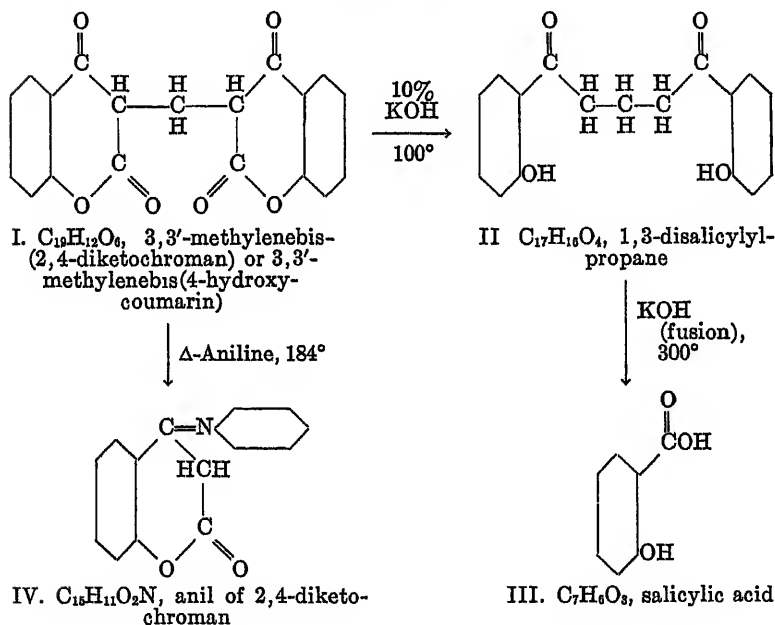
However, the ease of formation of the diketone suggests the decarboxylation of a β-keto acid structure. On this basis, together with the absence of phenolic properties in the hemorrhagic agent, the 2 carbon atoms and the 2 oxygen atoms which are eliminated in the formation of the diketone might be assigned to the position β to the carbonyls of the diketone and engaged in lactone formation with the phenolic hydroxyls. This postulation was given support by the following reaction.

Heating the hemorrhagic agent with phenylhydrazine resulted in cleavage to a compound containing 9 carbon atoms ascribable to the parent substance. Analysis of the product, m.p. 189–189.5°, indicated the formula $C_{21}H_{10}O_2N_4$ or $C_9H_4O_2(=N-\overset{H}{N}-C_6H_5)_2$. The recovery of a structural unit containing 9 atoms via degradation with phenylhydrazine suggested that this agent effected a cleavage of the parent substance $C_{19}H_{12}O_6$ into two C_9 units, with a simultaneous loss of 1 carbon atom, and coupling of each of the C_9 units with 2 moles of phenylhydrazine. Precedent for an anomalous reaction of this type under the influence of phenylhydrazine is not common. But this reaction, in conjunction with the results from the alkaline degradations, led us to consider the properties and constitution of the known dicoumarin derivatives, particularly those containing a methylene bridge (5).

Mature consideration of all the properties exhibited by the hemorrhagic agent and our knowledge of its behavior in the degradation reactions portrayed above placed under strong suspicion the synthetic product, α-methylenebisbenzotetronsäure, $C_{19}H_{12}O_6$, reported by Anschütz in 1903 in conjunction with his classical studies on the benzotetronic acid group (6). The description by Anschutz of this compound was restricted to the melting point, given as 260° (with decomposition), its insolubility in water, solubility in alkali, and recrystallization from benzene. The only reaction mentioned (but without experimental data) was its behavior toward aniline.

When the hemorrhagic agent was heated with aniline, the product formed proved to be the anil of 2,4-diketochroman (4-anilidocoumarin), $C_{15}H_{11}O_2N$, m.p. 262–263° (IV). The melting point of 4-anilidocoumarin reported by Anschütz (6) is 3° below that of our product. 18 of the 19 carbon atoms and all of the oxygen atoms in the hemorrhagic agent could be accounted for by the product recovered from this reaction (13.4 mg. of the hemorrhagic agent gave 18.2 mg. of the anil).

It should be emphasized that the melting point, 288–289°, of the hemorrhagic agent is almost 30° above that reported by Anschütz for the compound listed under the name α-methylenebisbenzotetronsäure. However, all of the degradation reactions of the hemorrhagic agent as well as all of its chemical properties

could be rationalized on the basis of the Anschütz compound. For the physiological activity of the hemorrhagic agent from spoiled sweet clover hay, there existed, however, no parallel (1, 2).

Synthesis of Hemorrhagic Agent from Spoiled Sweet Clover Hay
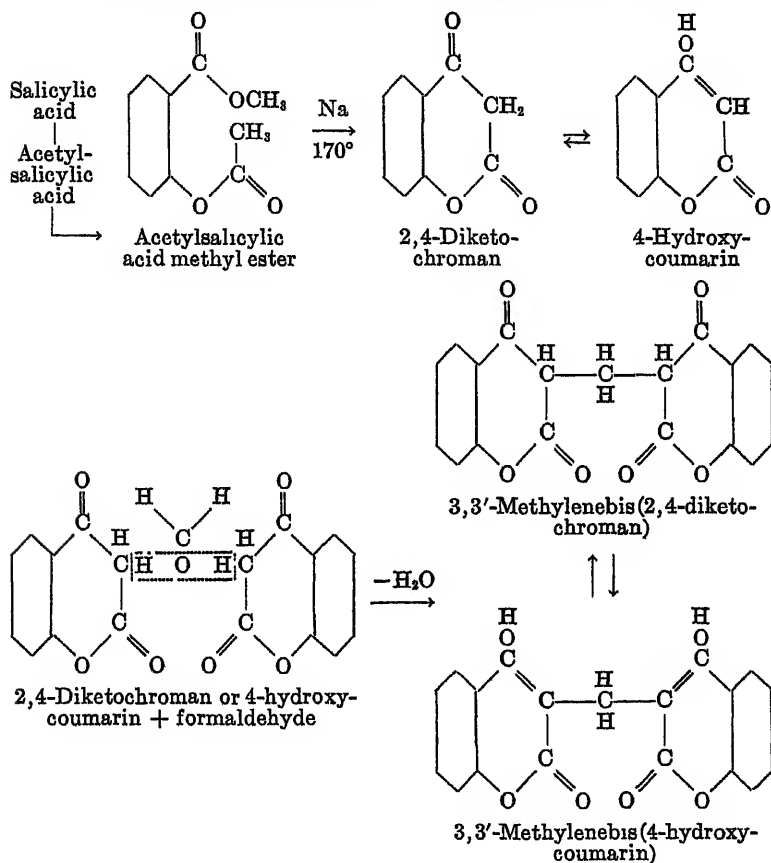
Accordingly, 4-hydroxycoumarin (benzotetronic acid) prepared from salicylic acid by the method of Pauly (7) was condensed with formaldehyde via the Anschütz procedure (6). No difficulty was experienced in realizing a synthetic product with a melting point in exact agreement with that of the pure naturally occurring hemorrhagic agent, 288–289°. All other physical

properties, crystal habit, limited solubility in the common solvents, and the absorption spectrum of the synthetic product were found to be identical with the naturally occurring substance (2). The analytical constants of the dimethyl ether and the diacetate prepared from the synthetic product coincided with those of the corresponding derivatives prepared from the natural material, and the same products (salicylic acid, the δ-diketone, the anil of 2,4-diketochroman) were recovered on degradation.

When the synthetic product was fed to our standardized assay rabbits at the levels previously reported for the natural substance, a comparable reduction in prothrombin level (or activity) was observed (1, 2, 8). Continued feeding of the synthetic product to rabbits, rats, guinea pigs, and dogs produced the fatal hemorrhages characteristic of the sweet clover disease (9, 10).

Since the origin of this exceptional substance in the spoiled sweet clover hays is the coumarin molecule, we prefer to have it designated as a derivative of coumarin, and accordingly suggest the use of the name 3,3'-methylenebis(4-hydroxycoumarin). It is apparent that tautomeric modification of 3,3'-methylenebis(4-hydroxycoumarin) into 3,3'-methylenebis(2,4-diketochroman) is possible. To this reversible transformation is ascribed, *inter alia*, the acidic properties of the substance, its behavior toward carbonyl reagents, and its degradation by alkali. Thus salt formation, methylation, and acetylation of the enol form of the substance would be expected. The cleavage caused by the organic bases phenylhydrazine and aniline may be considered a reverse aldol condensation. The mild alkali treatments effect opening of the lactone rings to produce a β-keto acid, which would decarboxylate readily to the δ-diketone. Stronger alkali effects cleavage of the double bond of the enol form of the diketone to produce salicylic acid (4). Accordingly in the formulations indicating the synthesis of 4-hydroxycoumarin and 3,3'-methylenebis(4-hydroxycoumarin) the keto structures are also indicated.

<div align="center">EXPERIMENTAL</div>

Mass Isolation of Hemorrhagic Agent (1, 2)—3 kilos of the spoiled hay were extracted with 30 liters of water at pH 3, steeped in 0.1 N sodium hydroxide, acidified to pH 3, filtered, and the residue extracted with two 20 liter portions of ethyl alcohol. The

alcoholic extract from 9 kilos of hay was concentrated at 25°, and the residue dissolved in 0.5 per cent sodium hydroxide and then acidified to pH 3. The precipitate was collected, suspended in 1 liter of methanol, and 2 liters of ethyl ether added. After filtration the methyl alcohol was removed by shaking with 6 liters of 2 per cent hydrochloric acid. The green ether solution was shaken with 36 per cent hydrochloric acid until the acid layer was just slightly colored. During this treatment additional ether was added to maintain the ether volume above 2 liters. The ether was concentrated to 500 ml. at atmospheric pressure, then to a thin syrup at reduced pressure. On examination in polarized light under high magnification, this syrup showed the presence of microscopic crystals of the hemorrhagic agent embedded in a matrix of amorphous material. Solubility tests indicated that the inert materials could be removed selectively.

The syrup was suspended by shaking with 200 ml. of methanol and centrifuged. The methanol layer was decanted and the solids resuspended in 200 ml. of methanol by shaking. This operation was repeated until the methanol washings were only slightly colored.

The remaining solids were transferred to a 50 ml. centrifuge tube and washed with a 10 per cent solution of Skellysolve A in methanol. This operation was repeated several times with methanol containing increasing concentrations of Skellysolve A. The progress of the washing was followed by observing the appearance of the crystals under polarized light. Recrystallization from benzene or cyclohexanone yielded macroscopic crystals of the crude product. After the final recrystallization from cyclohexanone the melting point of 288–289° was obtained. Thirty successive extractions gave 1800 mg. of the pure hemorrhagic agent, the over-all recovery (based on the prothrombin assays) being between 66 and 73 per cent of the total quantity present.

Preparation of Diacetate, $C_{19}H_{10}O_6(OCCH_3)_2$—The hemorrhagic agent (28.3 mg.) was added to 1 ml. of a mixture of equal parts of pyridine and acetic anhydride at 28°. After 30 minutes the crude acetate was filtered off, pressed dry, and recrystallized from benzene. The acetate melts at 250–252° (decomposes). Yield 34.7 mg. or 98 per cent of theory.

Analysis of Diacetate—The acetyl groups could not be estimated

with the usual acid saponification procedures. But after saponification in alcoholic potassium hydroxide and acidification with concentrated sulfuric acid, successful estimations were realized in the Perkins apparatus.

$C_{19}H_{10}O_6(OCCH_3)_2$
Calculated. Acetyl 20.48,   C 65.71,   H 3.81
Found.   "   20 4, 20.0, " 65.80, 65.72, " 3 89, 3.81

Alkaline Degradation of Hemorrhagic Agent—In each of the three degradation conditions given below the reaction mixture was worked up by the usual procedures for neutral, acidic, and phenolic substances. The over-all procedure will be apparent from the fusion with solid potassium hydroxide (*a*).

(*a*) *Degradation with Solid Potassium Hydroxide*—The hemorrhagic agent (7.3 mg.) was heated with 0.5 gm. of potassium hydroxide in a nickel crucible at 300° until the starting product dissolved with the evolution of gas. The melt was then held at approximately 300° for 10 minutes. The fused mass was cooled and taken up in 5 ml. of water.

The basic solution was first extracted with 5 ml. portions of ethyl ether to remove the neutral products. These were nil. The ether-extracted aqueous solution was then made acid with concentrated hydrochloric acid and again extracted with ethyl ether. The ether solution was extracted with 10 per cent sodium bicarbonate (5 ml.). The bicarbonate solution was acidified and extracted with ethyl ether. The resulting ether extract was washed with water, and then concentrated to permit crystallization of the acidic fragments. Only salicylic acid, $C_7H_6O_3$, was recovered, which was characterized by its melting point, 158–159°, and methyl ester. Yield 6.1 mg. or 98 per cent of theory. Under the same conditions control experiments with 10 mg. of coumarin gave salicylic acid quantitatively.

The ether solution remaining after the bicarbonate extraction was washed with water and concentrated to dryness. The quantity of phenolic material was negligible.

(*b*) *Degradation with 30 Per Cent Alcoholic Potassium Hydroxide* —The hemorrhagic agent (6.2 mg.) was refluxed in 5 ml. of 30 per cent potassium hydroxide in 90 per cent methyl alcohol for 24 hours. The reaction mixture was worked up as given in

section (a). The neutral fraction was nil. The phenolic fraction yielded 4.0 mg. of a δ-diketone with a melting point of 98–100°, which rose to 101–102° after recrystallization from 95 per cent ethanol. Since this product appears again in degradation (c), further comment on it will be deferred.

The acidic fraction yielded 2.5 mg. of salicylic acid (m.p. 157–158°).

(c) *Degradation with Aqueous Sodium Hydroxide*—The hemorrhagic agent (11.5 mg.) was refluxed in 10 per cent aqueous sodium hydroxide for 40 hours. The neutral and acidic fractions were nil. The phenolic fraction gave 9.5 mg. of the δ-diketone, 1,3-disalicylylpropane, $C_{17}H_{16}O_4$, m.p. 101–102°. Yield over 95 per cent of theory.

Properties of δ-Diketone (4)—It is optically inactive, the ferric chloride test is positive in ethanol, and the Folin-Denis test is also positive. The ketone couples with diazotized *p*-nitraniline (red precipitate).

Analysis of δ-Diketone—
$C_{17}H_{16}O_4$. Calculated. C 71.83,   H 5.63,   mol. wt. 284
            Found.   " 71.75, 71 70, " 5 53, 5.60, "   " 280
                   (micro-Rast in camphor) (average)

The δ-diketone forms a diether, $C_{17}H_{14}O_2(OCH_3)_2$, m.p. 86–88°. On fusion with potassium hydroxide the diketone yields salicylic acid quantitatively. It therefore represents an intermediate degradation product between the hemorrhagic agent and salicylic acid. Details of the characterization of the δ-diketone as 1,3-disalicylylpropane through synthesis by two independent methods are given in Paper VI of this series (4).

Degradation of Hemorrhagic Agent by Heating in Aniline—The hemorrhagic agent (13.4 mg.) was heated with redistilled aniline (0.2 ml.) at 180° for 30 minutes. The reaction mixture was poured into dilute hydrochloric acid to dissolve the excess aniline. The crude anil (18.2 mg.) was filtered and washed first with dilute acid and then with water. After one recrystallization from 20 ml. of 95 per cent ethanol the melting point rose to 262–263°, which is 3° above the melting point reported by Anschütz (6) for the anil of 2,4-diketochroman (4-anilidocoumarin). Yield approximately 95 per cent of theory. A control synthesis of the anil

from 4-hydroxycoumarin was made on a macro scale.   The products have identical properties and composition.

Analysis of Anil—
$C_{15}H_{11}O_2N$.   Calculated.   N 5.83,   C 75.95,   H 4.64
                   Found.   " 5.78, 5.90, " 76.00, 76.03, " 4.70, 4 73

Degradation of Hemorrhagic Agent by Heating with Phenylhydrazine—The hemorrhagic agent (13.0 mg.) dissolved in 0.5 ml. of phenylhydrazine was heated at 135° for 30 minutes.   The reaction mixture was poured into 10 per cent hydrochloric acid, whereupon a red gummy oil separated, which was washed by decantation with the acid.   The reaction product was dissolved in ethanol and recrystallized (two times).   Yield 13.9 mg., m.p. 189–189.5°.

The same product was also made in a control synthesis from 4-hydroxycoumarin on a macro scale.   Anschütz (6) reported that three different products are realized when 4-hydroxycoumarin is heated with phenylhydrazine.   He listed one with a melting point of 186° containing 11.05 per cent nitrogen (one phenylhydrazine residue).   It appears from the analyses reported below that the product realized by us contains two phenylhydrazine residues, which might occupy positions 3 and 4 in the coumarin ring.

Analysis of Phenylhydrazine Derivative—$C_{21}H_{16}O_2N_4$
Calculated.   N 15.75,   C 70.75,   H 4.49,   mol. wt. 356
Found.   " 15.74, 15.68, " 70.60, 70.70, " 4.54, 4.59,   "   " 364
                   (micro-Rast in camphor)

Synthesis of 4-Hydroxycoumarin (Benzotetronic Acid) (7)—
Acetyl methylsalicylate (100 gm.) was heated to 165° on an oil bath in an open beaker.   Metallic sodium (12 gm.) was introduced into the melt (with stirring) over a period of 1 hour.   The temperature was maintained between 165–175° by cooling.   As the sodium compound separated, the melt thickened and finally solidified.   The mass was then ground, the unused sodium decomposed with methanol, and the product taken up in 500 ml. of water.   After filtration the 4-hydroxycoumarin was precipitated by acidifying with hydrochloric acid.   The product was washed with dilute acid, and recrystallized from hot water.   Yield 12 gm. (14 per cent of theory), m.p. 204–206°.   Repeated recrystallization raised the melting point to 210°.

*Synthesis of 3,3'-Methylenebis(4-Hydroxycoumarin) (6)—*5.0

gm. of 4-hydroxycoumarin were dissolved in 1.5 liters of hot water (99°) to which an excess of 40 per cent formalin was added. The crude product was collected and washed copiously with water. It was dried and recrystallized from cyclohexanone, m.p. 288–289°. Yield 4.7 gm. (91 per cent of theory). The melting point of a
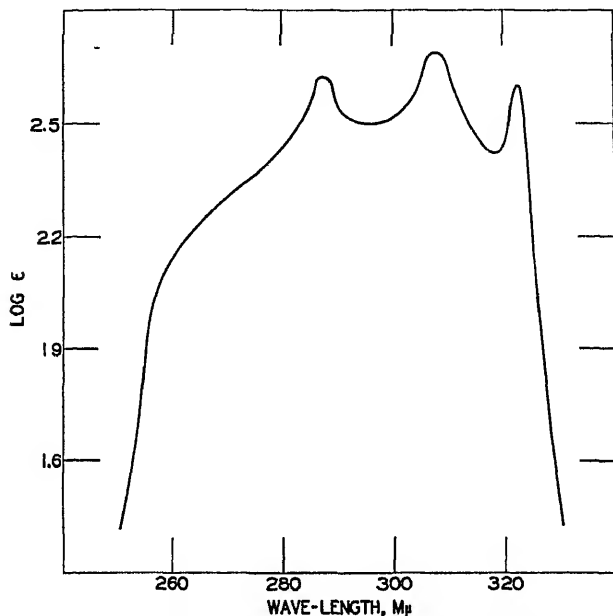
Fig. 1. Absorption spectrum of 3,3′-methylenebis(4-hydroxycoumarin) in cyclohexane; absorption maxima in mμ (log extinction values in parentheses), 288.0 (2.628), 308.5 (2.689), 323.5 (2.608).

mixture of the synthetic product and that isolated from hemorrhagic hay was 288–289°.

Analysis of Synthetic Product—$C_{19}H_{12}O_6$

Calculated. C 67.80,   H 3.60,   O 28.60
Found.   " 67.84, 67.80, " 3.49, 3 53, " 28.67 (by difference)

Electrometric Titration—The curve obtained with the synthetic product was so close to that of the natural product that the details need not be given again (2).

Absorption Spectra—The absorption curves obtained with the natural and the synthetic product were identical (see Fig. 1).

Physiological Activity (2, 8)—1.5 mg. doses of 3,3'-methylene-bis(4-hydroxycoumarin) fed to standardized susceptible rabbits (with relative clotting index values[2] of about 0.30 at the time of standardization) gave an index of about 0.50 after 24 hours and a relative clotting index of less than 0.10 after 40 hours, representative of an increase in prothrombin time from 25 to 28 seconds to 85 to 90 seconds. Continued feeding of the synthetic product to rabbits, rats, guinea pigs, and dogs first produced a prolonged prothrombin and whole blood clotting time followed by the hemorrhagic condition characteristic of the sweet clover disease, which eventually became fatal (9, 10). The details on the physiological activity of the substance at different dosage levels with various animals will be considered in a separate communication.

DISCUSSION

Relation of This Study to Development of Low Coumarin Strains of Sweet Clover—In previous publications from this station (11–14) it was pointed out that both of the undesirable aspects of sweet clover as a cultivated crop,[3] its unpalatability (bitterness) and the tendency of the hays to become hemorrhagic when improperly cured, appeared to have a common basis in the coumarin molecule. The elucidation of the structure of the hemorrhagic agent as 3,3'-methylenebis(4-hydroxycoumarin) substantiates this relationship, and suggests that the biological synthesis during spoilage involves an oxidation of the coumarin to 4-hydroxycoumarin followed by coupling with formaldehyde.

On this basis, the amount of coumarin in the sweet clover which was converted to the hemorrhagic agent in the spoiled hays employed in this study (1, 2) would be 0.0026 per cent (dry substance basis). The final coumarin content of these spoiled hays ranged from 0.75 to 1.58 per cent, which indicates that only a very small amount of the total coumarin was involved.

Reduction of the coumarin content by breeding or selection

---

[2] The relative clotting index is the ratio of the concentration of the normal plasma in the concentration range of 12.5 to 8.34 per cent to the concentration of the pathic plasma which gives the same clotting time (8).

[3] During the last three decades sweet clover in North America has risen from the status of a roadside weed to a place of importance as a forage crop (15).

(11, 12, 16) offers the possibility of improving palatability and reducing the economic hazard associated with the feeding of spoiled sweet clover hays or silage. But in view of the small amount of coumarin actually involved in the formation of hemorrhagic hays, and the limitations of the methods available at present for the estimation of coumarin (17), any strains of sweet clover selected by the plant breeder on the basis of the coumarin content as potentially desirable should be checked through actual spoilage tests, in which the prothrombin assay with standardized susceptible rabbits is used as the control determination (8).

3,3'-Methylenebis(4-Hydroxycoumarin) and Blood Coagulation Problem—Roderick pointed out that the hemorrhagic sweet clover disease is in a sense without parallel in animal pathology or human medicine (9, 10). Recently Quick (18) has affirmed this view in a résumé on the classification of hemorrhagic diseases due to defects in the coagulation mechanism of the blood and suggested that similar toxic agents may perhaps be encountered clinically which may attack the prothrombin of the blood.[4]

We should like to call attention to certain observations made with 3,3'-methylenebis(4-hydroxycoumarin) which might interest those dealing with the general problem of blood coagulation. The syndrome produced by feeding the substance to rabbits apparently does not result in permanent injury. Some of our assay rabbits have had the prothrombin level (or activity) reduced to 10 per cent, or below, of the normal over 100 times (10 day rest period between assays) without developing either indications of permanent injury, the acquisition of immunity, or increased susceptibility to the hemorrhagic agent (8). The administration of massive single doses (1.0 gm. to a 2.5 kilo rabbit or 5.0 gm. to an 8 kilo dog) effected a reduction in the prothrombin level (or activity) without producing gross signs of permanent injury. It appears that continued feeding of the substance is necessary for the production of hemorrhages.

In view of the prothrombin-reducing or inactivating properties of this dicoumarin, and the spread between the detectable and lethal dose, together with the relative ease with which it may be synthesized and administered, it would appear that its anticoagu-

[4] See also the recent comprehensive review on plasma prothrombin and vitamin K by Brinkhous (19).

lant properties merit consideration from the physiologist and hematologist (19, 20).

SUMMARY

1. Proof is presented through degradation reactions and by synthesis, that the hemorrhagic agent, $C_{19}H_{12}O_6$, m.p. 288–289°, present in improperly cured hay made from the common sweet clovers, *Melilotus alba* and *Melilotus officinalis*, is the dicoumarin, 3,3'-methylenebis(4-hydroxycoumarin).

2. The chemical and physical properties of the naturally occurring and the synthetic products have been shown to be identical.

3. The synthetic product has been shown to parallel the naturally occurring product in its capacity to reduce the prothrombin level (or activity) of standardized susceptible rabbits.

4. The hemorrhagic condition characteristic of the sweet clover disease has been produced in various species of experimental animals by continued feeding of the synthetic 3,3'-methylenebis(4-hydroxycoumarin).

5. The bearing of this study on the objectives of the plant geneticist to breed a low coumarin line of sweet clover and the possible value of the hemorrhagic agent to the physiologist and hematologist are discussed.

BIBLIOGRAPHY

1. Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *J. Biol. Chem.*, **136**, 47 (1940).
2. Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **138**, 21 (1941).
3. Tutton, A. E., The natural history of crystals, London, 42 (1924).
4. Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 529 (1941).
5. Simonis, H., Die Cumarine, Stuttgart (1916).
6. Anschutz, R., *Ber. chem. Ges.*, **36**, 465 (1903); *Ann. Chem.*, **367**, 169 (1909).
7. Pauly, H., and Lockemann, K., *Ber. chem. Ges.*, **48**, 28 (1915).
8. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941).
9. Roderick, L. M., *J. Am. Vet. Med. Assn.*, **74**, 314 (1929); *Am. J. Physiol.*, **96**, 413 (1931).

10. Roderick, L. M., and Schalk, A. F., *North Dakota Agric. Exp. Stat.*, *Bull. 250* (1931).
11. Brink, R. A., *Science*, **79**, 301 (1934).
12. Brink, R. A., and Roberts, W. L., *Science*, **86**, 41 (1937).
13. Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **119**, 269 (1937); *Ind. and Eng. Chem., Anal. Ed.*, **9**, 438 (1937).
14. Smith, W. K., and Brink, R. A., *J. Agric. Research*, **57**, 145 (1938).
15. *U. S Dept. Agric., Bur. Plant Ind., Bull. 1653* (1939).
16. Stevenson, T. M., and White, W. J , *Scient. Agric.*, **21**, 18 (1940).
17. White, W. J., and Horner, W. W., *Scient. Agric* , **21**, 29 (1940).
18. Quick, A. J., *Am. J. Med. Sc.*, **199**, 118 (1940).
19. Brinkhous, K. M., *Medicine*, **19**, 329 (1940)
20. Eagle, H , A symposium on blood and the blood-forming organs, Madison, 242–264 (1940).

.

STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

VI. THE SYNTHESIS OF THE δ-DIKETONE DERIVED FROM THE HEMORRHAGIC AGENT THROUGH ALKALINE DEGRADATION *

By CHARLES FERDINAND HUEBNER AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison)

In the previous communication it was shown that when the hemorrhagic agent, 3,3'-methylenebis(4-hydroxycoumarin), isolated from spoiled sweet clover hay is degraded with 10 per cent aqueous sodium hydroxide there is formed in practically quantitative yields a new δ-diketone, $C_{17}H_{16}O_4$, m.p. 101–102°, which was designated as 1,3-disalicylylpropane (1). This communication deals with the characterization of this δ-diketone by synthesis.

The recovery of this product in the phenolic fraction from the 10 per cent aqueous sodium hydroxide degradation indicated that it was a much weaker acid than the parent substance. This was substantiated by its insolubility in sodium carbonate and by the electrometric titration curve. These observations, together with the positive ferric chloride test (in ethanol), the formation of a dimethyl ether, $C_{17}H_{14}O_2(OCH_3)_2$, and the recovery of salicylic acid on fusion, indicated that the acidity exhibited by this substance might be ascribed to the presence of two phenolic hydroxyl groups.

The presence of carbonyl groups was initially indicated by reaction with phenylhydrazine but the crystalline product recovered was not homogeneous. However, when allowed to react with

hydrazine hydrochloride and sodium acetate in the usual manner, a crystalline product was obtained, m.p. 252°, which analyzes for $C_{17}H_{16}O_2N_2$.[1] This reaction indicates the loss of 2 oxygen atoms which were probably present as carbonyl groups. With am-

I. Methyl o-methoxybenzoate

II. o-Methoxy-
benzoylacetic
ester (sodio
derivative)

$-CH_3OH$ / Na

HCNa   Condensation
with | CH_2I_2

IV. Methyl ether of 1,3-
disalicylylpropane

$-CO_2$
$-EtOH$
(hydrol-
ysis)

III. Intermediate dicarboxylic
ester (not isolated)

Ag_2O | CH_3I

V. 1,3-Disalicylylpropane

Δ
$\overleftarrow{AlCl_3}$

VI. Phenyl glutarate
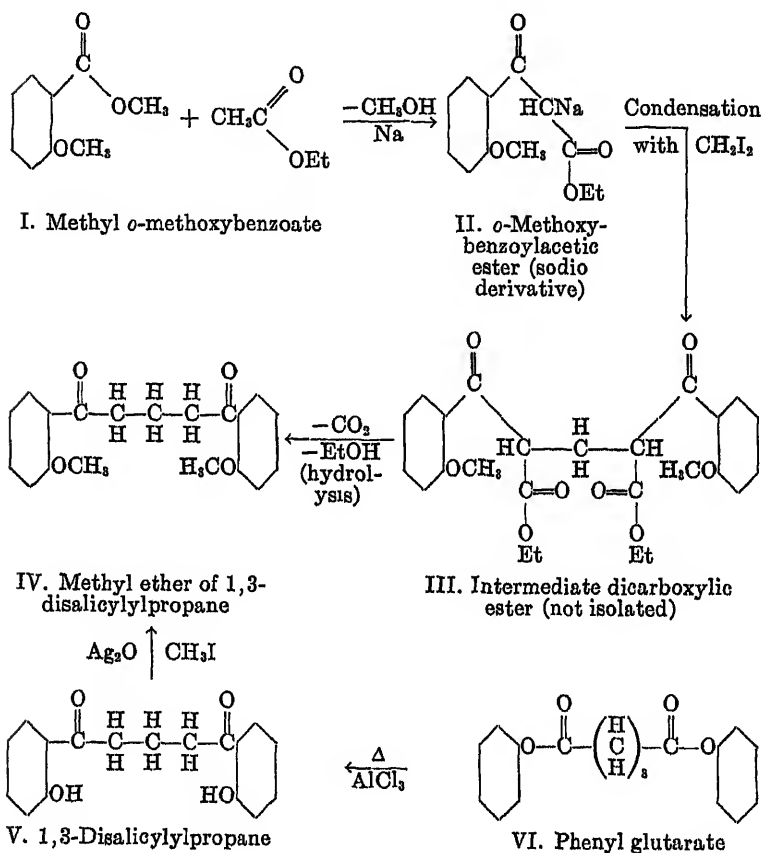
monium hydroxide the compound gives a yellow color. The formation of dihydropyridines from 1,5-diketones by reaction with ammonia has been recorded (2).

[1] While the structure of this compound (possibly a dihydropyridine derivative) has not been determined, it is of value as a characteristic derivative; work on it is in progress.

Mature consideration of the chemical properties of the ketone, together with the reported transformation of 4-hydroxycoumarin to o-hydroxyacetophenone under conditions that parallel those leading to the formation of the ketone (3, 4), suggested the structure assigned in formula (V). It appeared most practical to establish this structure by synthesis, since the 3 carbon atoms lost when it was degraded to salicylic acid were not recovered (1).

One synthetic approach was offered through condensation of o-methoxybenzoylacetic ester (II) with methylene iodide, the former being available from methyl o-methoxybenzoate (I), via the Claisen reaction. By subjecting the intermediate dicarboxylic ester (III) to hydrolysis and decarboxylation, the methyl ether of 1,3-disalicylylpropane (IV) would result. Accordingly the methyl ether (IV), $C_{17}H_{14}O_2(OCH_3)_2$, m.p. 86–88°, was first prepared from the parent ketone (V) by methylation with methyl iodide in the presence of silver oxide. A product identical with this methyl ether was successfully synthesized as outlined below.

A second confirmation of the structure proposed was realized by subjecting phenyl glutarate (VI) to the Fries rearrangement, which gave the ketone, m.p. 101–102°, directly.

The yields obtained in the above syntheses were not elegant. However, the physical constants and analytical data of the products obtained (IV, V) are in complete harmony with the structures assigned. The relationship of the structure of 1,3-disalicylylpropane to the parent hemorrhagic agent is given in Paper V of this series (1).

<center>EXPERIMENTAL</center>

Preparation of Methyl Ether of 1,3-Disalicylylpropane, $C_{17}H_{14}O_2$-$(OCH_3)_2$—The ketone (0.2 gm.) was refluxed for 1 hour with 0.5 gm. of silver oxide and 1 ml. of methyl iodide. After 12 hours ethyl ether was added, whereupon the solids were filtered off and repeatedly extracted with ethyl ether. The combined ether solutions were extracted with 10 per cent sodium hydroxide to remove the minute quantities of acidic material present (unmethylated ketone, etc.). The ether containing the neutral fraction was concentrated to dryness. The impure product was taken up in a mixture of benzene and petroleum ether and fractionally crystallized to remove traces of low melting neutral

impurities. After recrystallization, the yield of the pure product, m.p. 86–88°, was approximately 50 per cent.

$C_{17}H_{14}O_2(OCH_3)_2$
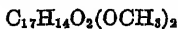
Calculated.	C 73.09,	H 6.41,	methoxyl 19.85
Found.	" 72 96, 72.98,	" 6 40, 6.41,	" 19 65, 20.05

Synthesis of o-Methoxybenzoylacetic Ester, $C_{12}H_{14}O_4$—This was prepared from methyl o-methoxybenzoate and ethyl acetate according to the method of Tahara (5). From a sample of the synthetic product the phenylpyrazolone was prepared, which melted at 130–131°. This is 17° higher than that reported by Tahara.

Synthesis of Methyl Ether of 1,3-Disalicylylpropane, $C_{17}H_{14}O_2$-$(OCH_3)_2$—The keto ester from above (4.27 gm.) in 5 ml. of benzene was allowed to react with 0.4 gm. of metallic sodium to form the corresponding sodio derivative. Methylene iodide (2.14 gm.) was then added. After the reaction mixture had stood for 12 hours, it was refluxed for 1.5 hours. The solvent was removed in a vacuum desiccator, and the resulting oil was taken up in 50 ml. of water. Carbon dioxide gas was passed through the solution and the unchanged methylene iodide was distilled off with steam. The residue was extracted with ethyl ether. The ether solution was concentrated and the resulting oil shaken for 15 hours with 2.5 ml. of 10 per cent sodium hydroxide. The alkali effects a simultaneous hydrolysis and decarboxylation to produce the desired product. A degradational cleavage also takes place which results in the formation of appreciable quantities of salicylic acid. The neutral materials were extracted from the alkaline solution with ether and the ether removed. The resulting syrup was seeded with crystals of the dimethyl ether prepared from the ketone realized in the 10 per cent sodium hydroxide degradation of the parent 3,3'-methylenebis(4-hydroxycoumarin) (1).

The crude synthetic dimethyl ether of the δ-diketone (0.35 gm. or 10 per cent yield) was first recrystallized from 95 per cent ethanol, and finally from a mixture of benzene and petroleum ether. The final product (0.22 gm.) melted at 86–88°. The melting point of a mixture of the synthetic product, and that prepared by methylating the parent diketone realized from the degradation of the hemorrhagic agent, showed no depression.

Analysis for $C_{17}H_{14}O_2(OCH_3)_2$ (Synthetic)—
    Found.   C 73.00, 72.98, H 6.40, 6.38, methoxyl 19.60, 19.85

The calculated values are given above.

Absorption Spectrum of Dimethyl Ether (Fig. 1)—The curves realized with the product prepared by methylating the ketone derived from 3,3'-methylenebis(4-hydroxycoumarin) and that obtained by synthesis coincide.

Synthesis of 1,3-Disalicylylpropane, $C_{17}H_{10}O_4$, by Fries Rearrangement—Phenyl glutarate (5.0 gm.) prepared by the method of Bischoff and von Hedenström (6) from glutaryl chloride (7) was added to a suspension of 5.24 gm. of aluminum trichloride in 10 ml. of carbon disulfide in a flask equipped with a stirrer, condenser, and thermometer well. The ester was added in 1 gm.
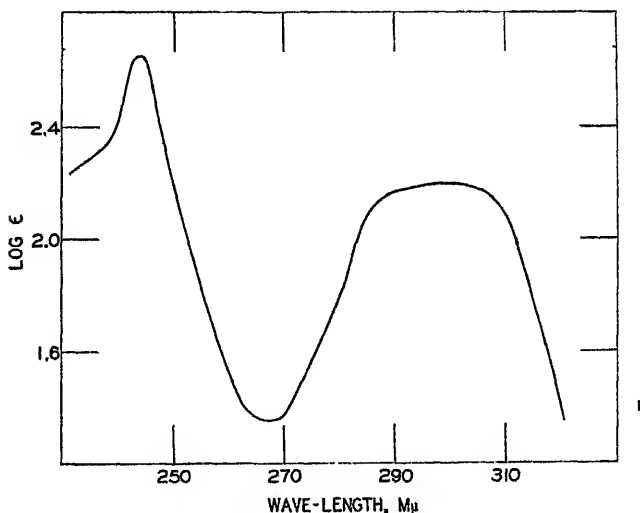
Fig. 1. Absorption spectrum of the dimethyl ether of 1,3-disalicylylpropane in cyclohexane; absorption maxima in mμ (log extinction values in parentheses), 244 0 (2.650), 294.7 (2 543).

portions to moderate the evolution of hydrochloric acid and the concomitant rise in temperature that results. Finally the reaction mixture was refluxed for 30 minutes. The product formed set up to a doughy mass. The carbon disulfide was distilled off and the temperature held at 175–180° for 2.5 hours. (The material does not melt.) Subsequently the desiccated mass was decomposed with 15 ml. of 30 per cent hydrochloric acid and 10 ml. of water (5°). The resulting tar was first extracted with ether and to the ether solution 10 per cent sodium hydroxide was added. Some solids separated which proved to be the sodium salt of the

diketone, which is but sparingly soluble in water. The solid matter was filtered off and added to the aqueous layer. After acidification with concentrated hydrochloric acid (dropwise) a crystalline product separated (3 gm.) which contained a mixture of the isomeric diketones formed in the Fries rearrangement.

It was possible to separate the desired isomer by six crystallizations from ethanol. The final product, 0.63 gm., melted at 100–102°, and the melting point of the mixture prepared with the corresponding diketone from the natural source showed no depression. The analysis and properties of the synthetic product and that realized from the 10 per cent sodium hydroxide degradation of 3,3'-methylenebis(4-hydroxycoumarin) are identical and need not be given again here (1).

SUMMARY

The δ-diketone, $C_{17}H_{16}O_4$, formed in the degradation of the hemorrhagic agent from spoiled sweet clover hay, 3,3'-methylene-bis(4-hydroxycoumarin), with 10 per cent sodium hydroxide is 1,3-disalicylylpropane. The δ-diketone was synthesized from phenyl glutarate via the Fries rearrangement. Its dimethyl ether, $C_{17}H_{14}O_2(OCH_3)_2$, was prepared by condensing o-methoxy-benzoylacetic ester with methylene iodide, and subjecting the intermediate dicarboxylic ester to hydrolysis and decarboxylation.

BIBLIOGRAPHY

1. Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 513 (1941).
2. Houben, J., Die Methoden der organischen Chemie, Leipzig, 3rd edition, **3**, 608 (1930).
3. Anschutz, R , *Ann. Chem.*, **379**, 336 (1911).
4. Simonis, H., Die Cumarine, Stuttgart (1916).
5. Tahara, Y., *Ber. chem. Ges.*, **25**, 1306 (1892).
6. Bischoff, C. A., and von Hedenström, A., *Ber. chem. Ges.*, **35**, 4085 (1902).
7. Reboul, M., *Ann. chim. et physiq.*, **5**, 304 (1878).

THE COLORIMETRIC DETERMINATION OF LACTIC ACID IN BIOLOGICAL MATERIAL

By S. B. BARKER AND WILLIAM H SUMMERSON

(*From the New York Hospital and the Departments of Medicine and of Biochemistry, Cornell University Medical College, New York City*)

The trend of recent research in the field of cellular and tissue metabolism has emphasized the importance of methods for the precise determination of small amounts of lactic acid. The quantities of lactic acid which are encountered when tissue slices, suspensions, and extracts are used for studies in metabolism are ordinarily below the range of the standard titrimetric lactic acid method of Friedemann, Cotonio, and Shaffer (1), which requires a minimum of about 0.5 mg. of lactic acid for the usual determination in duplicate. Of the various colorimetric methods which have heretofore been available for the determination of small amounts of lactic acid, only those of Mendel and Goldscheider (2) and Miller and Muntz (3) are sufficiently sensitive to be of possible value in this connection.

Both the Mendel-Goldscheider and Miller-Muntz methods depend primarily upon the reaction of Denigès (4), by which lactic acid is converted quantitatively into acetaldehyde on being heated with concentrated sulfuric acid. Mendel and Goldscheider determine the acetaldehyde colorimetrically by virtue of its reaction with veratrole (*o*-dimethoxybenzene), while Miller and Muntz utilize the reaction between acetaldehyde and *p*-hydroxydiphenyl which was first described by Eegriwe (5).

The reaction between *p*-hydroxydiphenyl and acetaldehyde is fundamentally far more sensitive than is the veratrole reaction. Nevertheless, in our study of the applicability of the two reagents to the determination of lactic acid in tissue metabolism preparations we were at first completely unable to obtain as satisfactory results with *p*-hydroxydiphenyl as had been obtained with a

modification of the veratrole procedure.[1] The variable results by the Miller-Muntz method, under presumably well controlled conditions, led us to suspect that an unknown factor was involved in the reaction. It was soon found that the reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid is markedly influenced by the presence of certain inorganic ions, notably those of iron, copper, and cerium. Other ions such as those of lead, mercury, nickel, and cobalt are without effect, and we are thus unable to confirm the finding of Block and Bolling (7) that lead influences the color reaction.

The effect of iron ions on the reaction is quite significant and points toward the explanation for the phenomenon which is offered later. While the presence of neither ferrous ion nor ferric ion alone is of analytical value, a suitable mixture of the two results in a final color which is from 3 to 5 times as intense as when the reaction is carried out in the ordinary way as described by Miller and Muntz, and the specificity of the reaction is also increased. The disadvantage of iron as a promoter of the color reaction lies in the fact that variations in the ratio between ferrous and ferric iron markedly influence color development, as is shown later, and an excessive amount of ferric iron produces a green color with the reagent alone.

The effect of copper is quite different from that of iron. The addition of cuprous ion is not necessary, since added cupric ion alone enhances color development. As the cupric ion concentration is increased, color enhancement increases until it becomes almost as great as that obtained with the optimal mixture of ferrous and ferric iron. Higher concentrations of cupric ion have no further effect, nor does the ion produce a color with the reagent alone. Cerium behaves similarly to copper, but offers no advantages over the latter.

We have therefore devised a colorimetric procedure for the determination of small amounts of lactic acid which incorporates the findings just described. The various steps in the procedure

---

[1] Barker (6). When this report was presented at New Orleans in March, 1940, the essential details of the p-hydroxydiphenyl method described here, including the use of copper and of an aqueous alkaline solution of the reagent, were also presented orally but are not included in the published abstract.

include (*a*) treatment of the protein-free sample by the copper hydroxide-calcium hydroxide procedure of Van Slyke (8) to remove interfering material; (*b*) conversion of lactic acid to acetaldehyde by heating in concentrated sulfuric acid in the proportion recommended by Mendel and Goldscheider; (*c*) color development by treatment in the concentrated acid solution with *p*-hydroxydiphenyl in the presence of added cupric ions. The *p*-hydroxydiphenyl is added as an aqueous alkaline solution rather than in the solid form recommended by Miller and Muntz.[2]

The procedure described here has been applied with satisfactory results to a large number of samples of whole blood, serum, saliva, tissue filtrates, and tissue hydrolysates. The sensitivity of the procedure is such that color development is ordinarily carried out on a portion of the sample which contains not more than 5 to 10 γ of lactic acid. This represents 1 cc. of a 1:50 or 1:100 dilution of the usual sample. Since the final volume of colored solution under the conditions described here is about 7 cc., it would appear possible to apply the method to much smaller amounts of lactic acid than are specified above, by the suitable use of smaller volumes and microphotoelectric colorimetry.

<div align="center">EXPERIMENTAL</div>

Reagents—

20 per cent solution of $CuSO_4 \cdot 5H_2O$.

4 per cent solution of $CuSO_4 \cdot 5H_2O$.

Solid calcium hydroxide, C.P., powdered.

Sulfuric acid, concentrated, sp. gr. 1.84.

1.5 per cent solution of *p*-hydroxydiphenyl in 0.5 per cent NaOH.

Removal of Protein—The solution undergoing analysis must be free from protein. Protein may be removed by any of the common procedures in which trichloroacetic acid, tungstic acid, zinc hydroxide, or cadmium hydroxide is employed. This was established by comparative analyses on blood samples which had been deproteinized by various procedures. In one such series of analyses a blood sample which contained 21.8 mg. per cent of lactic

---

[2] While this article was in preparation, the report of Koenemann (9) appeared, in which the use of an aqueous alkaline solution of *p*-hydroxydiphenyl for the Miller-Muntz procedure was proposed.

acid by the titrimetric Friedemann method gave colorimetric values of 21.7, 21.1, and 21.9 mg. per cent after deproteinization by trichloroacetic acid, tungstic acid, and zinc hydroxide respectively. Lactic acid added to the samples before precipitation of the proteins, in amounts equivalent to from 5 to 20 mg. per cent, was recovered quantitatively in the filtrate from all three precipitants. Similar results have been obtained with cadmium hydroxide. The precipitated protein should be removed by centrifugation rather than filtration, to avoid possible contamination from the filter paper.

Treatment with Copper and Calcium—A portion of the protein-free fluid is treated with 20 per cent copper sulfate solution and solid calcium hydroxide in the proportion recommended by Van Slyke (8). A convenient procedure is as follows: 1.0 to 5.0 cc. of protein-free filtrate, containing from 20 to 100 γ of lactic acid . (*i.e.*, 2.0 cc. of a 1:10 blood filtrate, from a resting subject), are added to 1 cc. of 20 per cent copper sulfate solution and made to a total volume of 10.0 cc. with water. Approximately 1 gm. of powdered calcium hydroxide is added and the mixture immediately shaken vigorously. The mixture is allowed to stand at room temperature for at least half an hour, with occasional shaking, and then centrifuged. The supernatant fluid is used for the final color development as described below. In removing the aliquot for analysis, care should be taken to avoid including any of the solid material which is usually present in the surface film. The aliquot is pipetted out from beneath the surface film, and the outside of the pipette is then wiped clean.

With certain types of material, the treatment with copper and calcium described above has been found suitable for the removal not only of glucose and other interfering substances but also of protein, thus eliminating the need for a separate deproteinization. For instance, a lactate solution containing 5 per cent of protein and 200 mg. per cent of glucose gave the same results on a direct 1:100 dilution of the original sample by the copper-calcium treatment as were obtained by the usual 1:10 deproteinization and subsequent 1:10 dilution through the copper-calcium procedure. Saliva has been analyzed satisfactorily by this method, as have acidified tissue extracts and acid tissue hydrolysates. The method is not applicable to whole blood, however, since there is a substance in blood corpuscles which interferes with the lactic acid determina-

tion and which is not removed by the copper-calcium treatment alone but is readily removed by any of the common methods of deproteinization. The nature of this substance is under investigation.[3]

Excessively acid or alkaline solutions should be approximately neutralized before treatment with copper and calcium. If the bright blue color of cupric hydroxide is not obtained when the mixture is shaken after the addition of calcium hydroxide, more calcium hydroxide must be added. It is also possible to carry out the copper-calcium procedure on a 1:5 or even a 4:5 dilution of the protein-free filtrate, but the amounts of copper sulfate solution and calcium hydroxide should not be changed.

Color Development—Transfer 1.0 cc. of the supernatant fluid from the copper-calcium treatment, containing between 2 and 10 γ of lactic acid, to a fairly wide test-tube (18 to 23 mm. inside diameter) and add 0.05 cc. of 4 per cent copper sulfate solution. Run in from a burette exactly 6.0 cc. of concentrated sulfuric acid, mixing the contents of the tube while the acid is being added. It is not necessary to keep the mixture cold. With adequate mixing the temperature of the solution does not rise above 70–80°. After the acid has been added, place the tube upright in a boiling water bath and allow it to remain for 5 minutes. Remove and place in cold water to cool to below 20°.

When the contents of the tube are sufficiently cool (but not before), add from a pipette exactly 0.1 cc. of the alkaline solution of *p*-hydroxydiphenyl. Disperse the precipitated reagent as quickly and uniformly as possible through the solution, and place the tube in a beaker of water at 30°. Allow to stand for 30 minutes (longer does no harm), then place the tube in boiling water for 90 seconds, remove, and cool in cold water to room temperature.

It is good practice to redisperse the precipitated reagent through the solution by gentle shaking at least once during the 30 minute incubation period. The 90 second heating in boiling water dissolves excess reagent, leaving a clear solution.

Color Measurement—Transfer the colored solution to a suitable container and read in a photoelectric colorimeter, using a filter which has a peak transmission at about 560 mμ. This filter was

[3] This material has now been identified as acetaldehyde (16).

selected on the basis of the spectrophotometric data of Block and Bolling (7), and has proved completely satisfactory. Because of the composite nature of the color (*i.e.*, appreciable light absorption in various portions of the spectrum), it is important that a filter with a spectral band not over 50 to 60 mμ wide be used. Both the Rubicon No. 565 and the Klett No. 56 filters fulfil this condition.   ·

For the initial setting of the colorimeter, either sulfuric acid alone or the reagent blank may be used. The latter procedure corrects the unknown reading for the value of the reagent blank, while the former does not. An alternative procedure for correcting for the value of the reagent blank is described in detail below.

Calculation of Results—The lactate concentration of the unknown may be obtained from the colorimeter reading by reference to a previously prepared calibration curve relating colorimeter readings and known concentrations of lactate. If such a curve is to be used, the standard lactate solutions should be put through the copper-calcium procedure before the color development is run. It is also obvious that the analytical conditions must be rigorously controlled with respect to every detail if future analyses are to be related to a previously prepared calibration curve.

A more convenient procedure for obtaining results is to make use of the strict adherence to Beer's law by the analytical system under the conditions described above. With the Evelyn (10) colorimeter (and also others equipped with a 0 to 100 linear scale and employing an equivalent depth of solution), a straight line is obtained over a concentration range from 0 to about 5 γ of lactate per cc. if instead of plotting the galvanometer reading G against lactate concentration, the value of $2 - log\ G$ is used. This line passes through the origin when a reagent blank tube is used for adjusting the colorimeter to the 100 reading, and the $2 - log\ G$ values then become directly proportional to the lactate concentration. The proportionality factor is readily established by careful analysis of known lactate solutions, and once it is known, the lactate concentration of an unknown solution is obtained by multiplying its $2 - log\ G$ value by the proportionality factor.

With the Summerson (11) colorimeter, the logarithmic scale makes the calculation of results somewhat simpler, since the scale

readings themselves are directly proportional to concentration for systems which adhere to Beer's law. With the reagent blank tube set at 0, the scale reading for a standard lactate solution divided into the lactate concentration gives the proportionality factor directly.
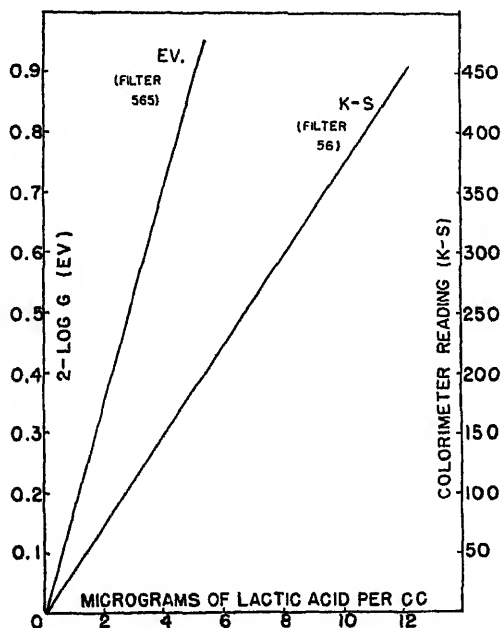
FIG. 1. Range of proportionality between color intensity and concentration of lactic acid, as obtained with the Evelyn (*EV*) and Klett-Summerson (*K-S*) photoelectric colorimeters. For the Evelyn colorimeter, color intensity is proportional to $2 - log\ G$, where G is the galvanometer reading. The reason for the differences in slope and range between the two colorimeters is given in the text.

The range of proportionality for both the Evelyn and Klett-Summerson photoelectric colorimeters is shown in Fig. 1. The data were obtained by the analysis of solutions of known lactate content, prepared from both lithium lactate and zinc lactate. For the Evelyn colorimeter the data are expressed in terms of the $2 - log\ G$ values, while for the Klett-Summerson colorimeter the scale readings are plotted directly. Both curves have been corrected for the reagent blank values.

The difference in slope between the two curves is due to the use of narrower test-tubes in the Klett-Summerson instrument than are used in the Evelyn instrument. The range of satisfactory proportionality is therefore greater for the former colorimeter, but the sensitivity in terms of scale divisions per microgram of lactate is correspondingly less. For a particular colorimeter and color filter, the slope of the curve, and hence the proportionality factor, is reasonably constant. Any serious deviation from previous values should be reinvestigated.

The desirability of calculating results with the photoelectric colorimeter in terms of the reading of a simultaneously prepared standard instead of by reference to a previously prepared calibration curve has been discussed elsewhere (11). In the present instance, this may be done by running a standard and a reagent blank along with each series of unknowns, and calculating the proportionality factor as described above. An alternative procedure, which has been much practiced and which does not make the accuracy of the results depend upon the precision with which the relatively small reagent blank may be established, is to omit the reagent blank determination and to run two standards, at different levels, in each series of unknowns. The difference in reading between the two standards enables the proportionality factor to be calculated, and from this and the reading of either standard the "apparent blank" is obtained. An example of this calculation is given below. The data are in terms of the readings on the Klett-Summerson colorimeter, but the principle is equally applicable to instruments with which the $2 - log\ G$ value must be used.

Example

Reading of unknown = 170
  "   " 2 γ standard = 95
  "   " 4 "   "   = 175
Therefore 2 γ = 80 scale divisions
  and 1 " = 40 "   "
"Apparent blank" = 95 − (2 × 40) = 15
Unknown reading minus blank = 170 − 15 = 155
Lactate concentration of unknown = 155/40 = 3 88 γ

Proof of Method—The three commonly accepted criteria of an analytical procedure are that the values obtained correspond well

with those obtained by other procedures, that added material is completely recovered, and that the procedure has a high degree of specificity for the substance in question.   Evidence bearing on these points is presented below.

Fig. 2 shows a comparison on a variety of samples between the colorimetric method described here and the titrimetric method
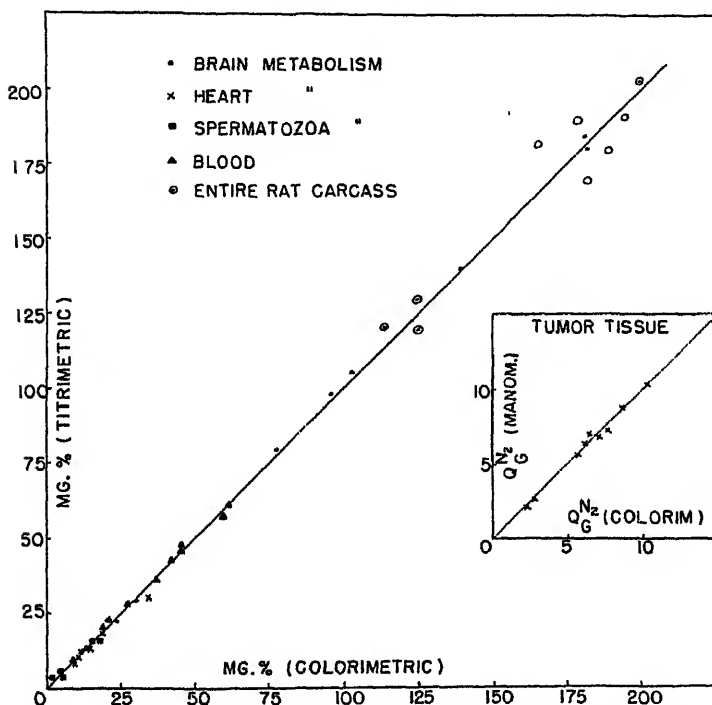
FIG. 2. Comparative lactic acid values on a variety of samples, as obtained by the authors' colorimetric method and by the titrimetric method of Friedemann et al. (main part of figure) or the manometric method of Warburg (inset).

of Friedemann, Cotonio, and Shaffer (1).   The agreement is within ±5 per cent in general, with a few values falling in the ±10 per cent range.   It should be pointed out, however, that in our experience the latter is the range of reliability of the titrimetric procedure, and may not entirely represent a discrepancy between the amount of lactic acid as determined by the two methods.

The inset in Fig. 2 shows a similar comparison between the

colorimetric method and the manometric method of Warburg
(12), as applied to the determination of the lactic acid production
by tumor tissue in the presence of glucose under anaerobic condi-
tions.   The quantities of lactic acid concerned here were too small
for analysis by the titrimetric method.   By the manometric
method acid production is measured in terms of the decomposition
of bicarbonate, and the method is not specific for lactic acid.   How-
ever, Negelein (cf. Warburg (12)) has shown that the only acid
which comes into question under these conditions is lactic acid.

TABLE I

Recovery of Added Lactic Acid

Tissue filtrate	No. of analyses	Lactic acid added	Lactic acid originally present	Lactic acid recovered	
		mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*
Brain	4	20 0	25	20.3	102
	4	50.0	25	50.9	102
	4	50.0	180	52.3	105
Heart .	4	25.0	10– 35	25 3	101
	4	50.0	10– 35	49.4	99
	4	75 0	10– 35	74.3	99
Entire rat carcass	3	50.0	150–200	50.7	101
(total carbohydrate	8	100.0	150–200	100.0	100
hydrolysate)	6	200 0	150–200	207 5	104
Blood, dog. .	3	4.0	25	4 0	100
	10	5.0	10	5.0	100
	6	10.0	10– 15	10.1	101
	3	15.0	60	15.2	101
	6	20.0	10– 15	18.9	95
Blood, cat. . . .	3	5.0	20	4.9	98
	3	10.0	15	9.7	97

The agreement between the colorimetric and manometric methods
is just as definite as with the titrimetric method.

The recovery of lactate added to a variety of analytical samples
is shown in Table I.   Quantitative recovery of amounts from 4
to 200 mg. per cent was obtained, even when the added lactate
was as little as one-fourth of that already present.   This fully
covers the concentration range encountered under most condi-
tions, but undoubtedly even higher values can be handled satis-
factorily by proper dilution.

The results of specificity tests on a considerable number of organic compounds are summarized in Tables II and III. For these tests, a solution of the substance containing 25 γ per cc. was examined with regard to both the production of a significant color under the analytical conditions and interference with the determination of lactate. This concentration is from 5 to 10

TABLE II

Substances Which Do Not Interfere with Analysis for Lactic Acid under Conditions Specified in Text

Acetic acid	Ethyl alcohol	Lysine
Acetoacetic acid	Ethylene glycol	Malonic acid
Acetone	Fructose	Maltose
Adenine	Fumaric acid	Mannose
Alanine	Galactose	Melezitose
Allantoin	Glucose	Ornithine
α-Aminobutyric acid	Glucuronic acid	Oxalic acid
Arabinose	Glutamic acid	Phenylalanine
Arginine	Glutathione	Phosphoglyceric acid
Ascorbic acid	Glycerol	Proline
Asparagine	α-Glycerophosphate	Raffinose
Aspartic acid	β-Glycerophosphate	Sarcosine
Caffeine	Glycine	Serine
Carnosine	Hexose diphosphate	Succinic acid
Choline	" monophosphate	Tartaric "
Citric acid	Histidine	Threonine
Creatine	Homocystine	Trehalose
Creatinine	β-Hydroxybutyric acid	Tryptophane
Crotonic acid	Hydroxyproline	Tyrosine
Cystine	Hypoxanthine	Urea
Diphosphoglyceric acid	α-Isohydroxybutyric	Uric acid
Dulcitol	acid	Valine
Ergothioneine	Isoleucine	Xanthine
	Leucine	Xylose

times greater than that of lactate in the usual aliquot taken for color development, and is equivalent to a concentration of 250 mg. per cent at a 1:100 dilution.

The compounds were selected from the point of view not only of their possible occurrence in biological fluids but also of their use as experimental variables in tissue metabolism studies. The data of Tables II and III demonstrate that the complete analytical

procedure is highly specific for lactic acid, if the copper-calcium treatment is routinely included. Of the compounds which are listed as not interfering with the method, in Table II, many give significant amounts of color if the copper-calcium treatment is omitted, but all are adequately removed by this treatment under the conditions specified. It must be mentioned, however, that

TABLE III

Interfering Substances Which Copper-Calcium Treatment Will Remove Only at Low Concentration or Will Not Remove at All

Compound	Color obtained	Maximum allowable amount for complete removal, γ per cc. final solution	Color yielded by 1 γ in terms of γ of lactic acid
Removed at low concentration			
Dihydroxyacetone	Similar to lactate	1	0.02 *
Glyceric aldehyde . .	" " "	10	0 005
p-Hydroxyphenyllactic acid .	" " "	15	0.005
Malic acid . . .	" " "	5	0.01
Pyruvic acid . .	" " "	5	0.025
Rhamnose	" " "	5	0 02
Not removed at all			
Acetaldehyde . .	Similar to lactate		2.05
Acrolein	" " "		0.10
Methylglyoxal	" " "		0.80
Propylene glycol	" " "		0.16
Djenkolic acid	Blue-green		
Formaldehyde . . .	"		
α-Hydroxybutyric acid ..	Blue-gray		
Methionine .. .	(See text)		

the copper-calcium treatment appears to depend for its effectiveness in some instances on a process of adsorption rather than precipitation, and the completeness of removal at higher concentrations than that specified should be established by experiment.

This is illustrated by the data of Table III, which lists those substances which the copper-calcium treatment will remove completely at low concentrations only, or will not remove at all. For

substances in the former class, the limiting concentration for complete removal is given, as well as the color value in terms of lactate.   From these data the extent of possible interference under particular experimental conditions may be evaluated.

Those interfering substances which are not removed at all by the copper-calcium treatment are, with the exception of methionine and propylene glycol, readily distinguishable from lactate in that they either react directly with p-hydroxydiphenyl in the cold (which lactate does not) or they yield a final color which is distinctly different from the characteristic violet of the acetaldehyde reaction.   The question of their occurrence in an analytical sample may therefore be readily answered.   No interference of this type has ever been noted in biological samples except in the case of the substance present in red blood cells which was mentioned above and which was detected because of its ability to yield a color in the cold.

Propylene glycol is indistinguishable from lactate except for its relative color intensity per microgram, as given in Table III.   As far as we know, the presence of significant amounts of propylene glycol in biological material is not to be expected.   If it has been added to the system as an experimental variable, it will interfere to the extent indicated by its relative color value.

The effect of methionine on the analytical procedure is quite unusual.   In the presence of small amounts of free methionine, lactate (and acetaldehyde) solutions give definitely *less* color than expected.   The decrement in color is proportional within certain limits to the amount of methionine present; amounts of the same order of magnitude as the lactate present are readily detected, and larger amounts lead to complete inhibition of color formation. The nature of this interference is still under investigation, but it appears at the present time to be due to a reaction in concentrated sulfuric acid between methionine (or decomposition products) and acetaldehyde, to produce a substance which does not react with p-hydroxydiphenyl.   The reaction appears to be highly specific for methionine, and is at present being used as the basis of a method for the microdetermination of methionine.   The details of this method will appear shortly.

From the point of view of lactic acid determination, interference by methionine is not regarded as significant under ordinary condi-

tions, since the occurrence of free methionine in a sample in amounts equivalent to lactate would be rather unusual. Furthermore, neither the titrimetric nor the manometric lactic acid method is interfered with in a similar manner by methionine, and the degree of correlation between these methods and the colorimetric method which has already been shown in Fig. 2 indicates that the colorimetric method gives accurate results under all ordinary conditions.

<div align="center">DISCUSSION</div>

Analytical Precautions—Few precautions with regard to the reagents appear to be necessary. The sulfuric acid should be of reagent quality; acid from a number of different sources has proved equally satisfactory. The acid should be protected from contamination by organic matter while standing in the laboratory, and since the amount of acid must be measured exactly during the analysis, it is best dispensed from a burette. It is convenient to arrange the burette so that it may be filled directly from the acid bottle by suction, or a burette with an attached reservoir may be used. All stop-cocks which come in contact with acid must be free from grease, so that a "greaseless" stop-cock or an ordinary one lubricated by a small amount of the acid itself must be used.

The Eastman Kodak Company grade of *p*-hydroxydiphenyl may be used directly without further purification by recrystallization. One recrystallization from alcohol or acetone removes material which gives a small amount of color in alkali, but no significant differences in analytical performance have been noted between the purified and unpurified material. The reagent solution is prepared by dissolving the dry material in 5 per cent or 10 per cent sodium hydroxide by stirring and warming and then diluting to a final alkali concentration of 0.5 per cent. The solution keeps indefinitely at room temperature in a stoppered brown bottle, and is conveniently dispensed from a pipette attached to the stopper and known to deliver 2 drops per 0.1 cc.

The U.S.P. or C.P. grades of calcium hydroxide are equally satisfactory for use in the preliminary copper-calcium procedure.

We do not find it necessary to cool the solution during the addition of the sulfuric acid, nor are stoppered tubes required during

the heating period which converts lactate to acetaldehyde. The time of heating at this stage is not critical, identical results being obtained with heating periods of from 3 to 10 minutes. Longer heating than 10 minutes may lead to slight losses. The 4 per cent copper sulfate solution is added to the system before heating merely as a matter of convenience, since it does not affect the conversion of lactate to acetaldehyde in any way.

The color reaction is not significantly affected by a slight excess of either the reagent or of copper ions. Color development is complete in 30 minutes at about 30°, and longer standing at this stage does not affect the results. The curve relating temperature and color intensity is almost flat between 20–30°, temperatures outside of this range giving less color. This is particularly true of temperatures above 35°, owing to rapid destruction of the reagent. It is therefore important that the solution be well cooled after the 5 minute heating period and before the addition of the reagent, as otherwise low results will be obtained. The color of the mixture during the 30 minute incubation period is usually a blue-violet, and the appearance of a red-violet color at this stage, coupled with the disappearance of the precipitated reagent, is indicative of too high a temperature.

Heating the solution for 90 seconds, after color development is complete, is for the purpose of clarifying the solution by destroying excess reagent, and stabilizing the color. Heating from 1 to 2 minutes at this stage does not affect results, but longer heating changes the spectral characteristics of the color, making it unsuitable for measurement at 560 mμ. After the heated solution has been cooled, the color is quite stable for some time, a decrease of only about 5 per cent being noticeable after 3 hours at room temperature.

A major source of error appears to be contamination of the sample by lactic acid itself, from the skin surfaces. It should be remembered that the standard solutions contain only a few parts per million of lactic acid. If a test-tube of distilled water is closed with the palm of the hand and shaken vigorously for a few minutes, the resulting solution will contain about as much lactate as such a standard. Duplicate analyses on a given copper-calcium filtrate are expected to agree within 2 per cent or less. When deviations greater than this have been obtained, and the analyses

repeated, the results have invariably indicated the presence of excess lactate as the source of error. Precautions against this type of contamination include the use of glass-stoppered vessels for aqueous solutions which are to be mixed or shaken, or the mouth of the vessel may be closed with a small square of Parafilm (fresh surface down) held in place by the finger tip. For wiping off the outside of pipettes, individual pieces of fresh cleansing tissue are recommended, and glassware should not be drained in contact with surfaces which are apt to be contaminated by dust or perspiration. These precautions may appear to be extreme, but experience has demonstrated their value.

Other possible sources of error include inadequate mixing of the water and the viscous sulfuric acid during the analysis, and the presence of traces of chromic acid (from cleaning mixture) on the glassware. Glassware which has been cleaned with chromic acid should be passed through an alkaline wash solution (soapy water, calgonite) before being rinsed and dried. With regard to adequate mixing, the use of wide test-tubes as specified will aid in this respect. Particular attention should be paid to mixing well after the addition of the p-hydroxydiphenyl, since this substance is quite insoluble in the concentrated acid mixture and must be uniformly dispersed if quantitative results are expected.

Effect of Inorganic Ions on Color Development—The effect of inorganic ions on the color reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid has been studied in the light of the observation recorded above that, while neither ferrous nor ferric iron alone is of appreciable analytical value, a suitable mixture of the two enhances color development significantly. Furthermore, the amounts of iron or copper which are necessary for optimal color development are several hundred times greater than the amount of acetaldehyde present and approach the order of magnitude of the p-hydroxydiphenyl concentration. These facts indicate that the effect of added ions is primarily on the p-hydroxydiphenyl itself, and that the action involves oxidation-reduction equilibria of some kind.

We therefore carried out some measurements of the E.M.F. of the p-hydroxydiphenyl-sulfuric acid mixture against a platinum electrode in the presence of those ions which we found to be of significance. The results obtained are illustrated by the data

of Fig. 3. The left-hand section of Fig. 3 gives data on the relative color intensity and observed E.M.F. in the presence of varying proportions of ferrous and ferric ions. The right-hand section shows the influence of a single type of added ion, either ferrous, ferric, or cupric.
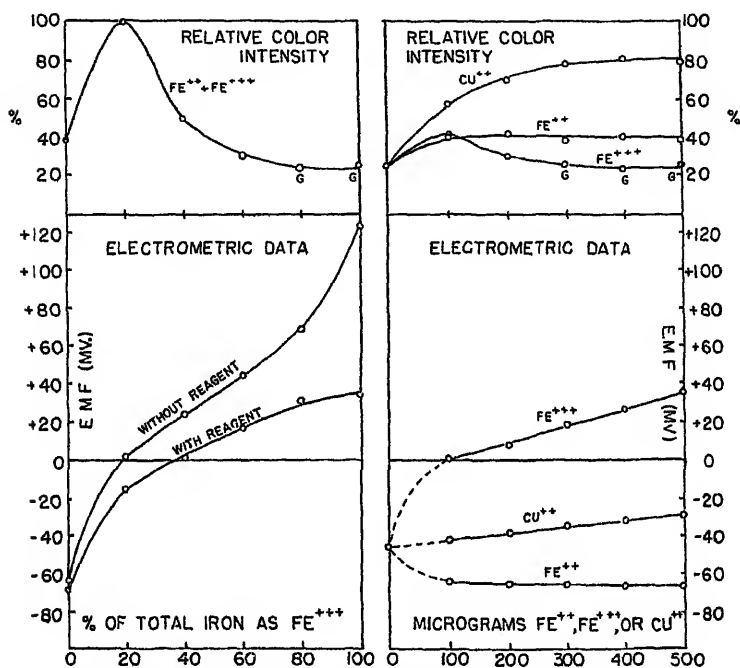
FIG. 3. The influence of added inorganic ions on the intensity of color obtained in the lactic acid method, and on the E.M.F. as measured against a platinum electrode. The left-hand chart gives data for mixtures of ferrous and ferric ions, while the right-hand chart refers to ferrous, ferric, or cupric ions alone. The symbol G refers to the appearance of a green color rather than the characteristic violet of the acetaldehyde reaction.

The electrometric measurements were made with a smooth platinum electrode, a mercurous sulfate-saturated potassium sulfate half-cell as reference electrode, and a vacuum tube voltmeter. The system investigated consisted of 6 parts of concentrated sulfuric acid and 1 part of water containing a few micrograms of acetaldehyde (prepared from lactate by previous treat-

ment with hot concentrated sulfuric acid) and the inorganic ion or ions to be studied, together with 1.5 mg. of p-hydroxydiphenyl in suspension. In one series of measurements the p-hydroxydiphenyl was omitted (curve labeled "without reagent" in Fig. 3). Liquid junction with the reference electrode was effected through a layer of saturated potassium sulfate solution over a restricted portion of the surface of the sulfuric acid-acetaldehyde mixture.

Readings on each solution were begun immediately after the p-hydroxydiphenyl was added and the liquid junction effected, and were taken at intervals until constancy within 1 millivolt was obtained. This ordinarily required from 10 to 30 minutes, depending upon the experimental conditions. Since equilibrium was reached almost immediately in the absence of p-hydroxydiphenyl, it is concluded that the potentials measured are determined to a certain extent by the rate of solution of the insoluble reagent. Despite this variable factor, readings under a given set of conditions were readily reproducible from day to day, even with regard to the rate of attainment of equilibrium. The least stable system electrometrically was that containing p-hydroxydiphenyl without added ions, the potentials in this case having varying initial values and rarely reaching even approximate constancy. The uncertainty with regard to this point in Fig. 3 is indicated by the broken sections of the curves in the right-hand portion of the figure.

The electrometric data are plotted in terms of the observed E.M.F. of the system rather than by reference to the normal hydrogen electrode, since neither the electrode reaction nor the liquid junction potential or pH of the solution is known. The difficulties associated with the precise interpretation of potentiometric measurement in concentrated acid solution have been pointed out by Michaelis, Schubert, and Granick (13).

Nevertheless, the data present certain significant points. We note a particular correlation between the observed values of the E.M.F. and the relative color intensity. Maximum color intensity with ferrous-ferric ion mixtures corresponds to an E.M.F. of between -10 and -30 millivolts, and it is when this zone is reached by the addition of cupric ion that color intensity in the presence of this ion corresponds to that obtained with ferrous-ferric ion mixtures. Neither ferrous nor ferric ion alone gives an E.M.F. falling within this zone, and the color intensity under these condi-

tions is proportionately low. In the absence of added ions, the E.M.F., while quite variable, as stated above, always passes through the zone in question, and this is probably why any color at all is obtained under these conditions.

The data also demonstrate why copper is superior to iron for color enhancement. In the presence of variable amounts of cupric ion the E.M.F. is relatively stable within the zone associated with maximal color values, even though the color obtained with copper is not quite as great as when an optimal mixture of ferrous and ferric iron is used, so that a slight excess or deficit of cupric ion is without significance. For the ferrous-ferric mixture, on the other hand, the problem of how to maintain the proper ratio between the two ions becomes of importance, and an excess of ferric ion will produce a green color rather than the characteristic violet of the acetaldehyde reaction.

We postulate therefore an oxidation-reduction equilibrium of some kind between p-hydroxydiphenyl and certain inorganic ions, with the color reaction between this reagent and acetaldehyde markedly dependent upon the maintenance of the proper equilibrium conditions. We plan a further study of this phenomenon, particularly with regard to defining more precisely the nature of the reactions involved, since the effect of inorganic ions noted here appears to be but one instance of a general condition. For instance, Dische and Laszlo (14) have found that cupric ions promote color development between hydroquinone and acetaldehyde in concentrated sulfuric acid; Winkler (15) enhances color development in the Hopkins-Cole reaction for tryptophane by the addition of cupric ions; and we have noted that the Hopkins-Cole reaction is likewise susceptible to color enhancement in the presence of a suitable mixture of ferrous and ferric ions.

SUMMARY

A method for the colorimetric determination of lactic acid in biological material is described, in which lactic acid is converted into acetaldehyde by treatment with concentrated sulfuric acid, and the acetaldehyde determined by its color reaction with p-hydroxydiphenyl in the presence of cupric ions. The color is read in a photoelectric colorimeter with a filter having a peak transmission at 560 mμ.

The method is applicable to a variety of biological material, after deproteinization by any of the standard procedures. Comparative analyses by other methods, as well as specificity studies on a large number of pure compounds, indicate that the procedure has a high degree of specificity for lactic acid. The sensitivity of the method is such that color development is carried out on a portion of sample containing not over 5 to 10 γ of lactic acid per cc., with differences of less than 0.1 γ per cc. readily detectable.

An electrometric study of the reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid indicates that this reaction is dependent upon the establishment of an oxidation-reduction equilibrium of some kind, and that optimal conditions for color development are only obtained in the presence of certain inorganic ions. Data are presented showing the relation between color development and the E.M.F. against a platinum electrode in the presence of these ions.

BIBLIOGRAPHY

1. Friedemann, T. E., Cotonio, M., and Shaffer, P. A , *J. Biol. Chem.*, **73**, 335 (1927).
2. Mendel, B., and Goldscheider, I , *Biochem. Z* , **164**, 163 (1925).
3. Miller, B. F., and Muntz, J. A., *J. Biol. Chem.*, **126**, 413 (1938)
4. Denigès, G., *Bull. Soc chim.*, **5**, 647 (1909).
5. Eegriwe, E., *Z. anal Chem.*, **95**, 323 (1933).
6. Barker, S. B , *Am. J. Physiol.*, **129**, P305 (1940).
7. Block, R J., and Bolling, D., *J. Biol. Chem* , **130**, 365 (1939).
8. Van Slyke, D D., *J. Biol. Chem* , **32**, 455 (1917).
9. Koenemann, R H., *J. Biol. Chem.*, **135**, 105 (1940).
10. Evelyn, K. A., *J. Biol Chem.*, **115**, 63 (1936).
11. Summerson, W. H., *J. Biol. Chem.*, **130**, 149 (1939).
12. Warburg, O., Über den Stoffwechsel der Tumoren, Berlin (1926); translated by Dickens, F., London (1930).
13. Michaelis, L., Schubert, M. P., and Granick, S., *J. Am. Chem. Soc.*, **62**, 204 (1940).
14. Dische, Z., and Laszlo, D., *Biochem. Z.*, **187**, 344 (1927).
15. Winkler, S., *Z. physiol. Chem.*, **228**, 50 (1934).
16. Barker, S. B., *J. Biol. Chem* , **137**, 783 (1941).

THE BIOLOGICAL ESTIMATION OF PYRIDOXINE
(VITAMIN B₆)*

By THEODORE W. CONGER AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

Since the discovery of vitamin B₆, several biological assay procedures for this factor have been proposed (György (1); Lunde and Kringstad (2); Wilson and Roy (3); Schneider *et al.* (4); Halliday and Evans (5); Edgar, El Sadr, and Macrae (6); Dimick and Schreffler (7); Reedman *et al.* (8)). These methods, with the exception of that of Edgar *et al.*, are based upon the cure of acrodynia produced on a vitamin B₆-deficient ration or upon a combination of growth and the cure of acrodynia of vitamin B₆-depleted rats. Edgar *et al.* propose an assay based on the growth response alone. In light of the fact that factors other than vitamin B₆ are concerned in the production and alleviation of dermatitis in the rat, methods involving the curing of dermatitis present difficulties in technique and interpretation (Birch (9); Schneider *et al.* (10)). Furthermore some workers have not been able to produce the dermatitis consistently (Dann (11)).

The method described here is based entirely upon the growth response of vitamin B₆-depleted rats supplemented either with synthetic pyridoxine or the substance to be assayed. Two rations are described. In Ration I a fullers' earth filtrate of a butanol extract of 1:20 liver concentrate powder is used to supply pantothenic acid and unidentified factors necessary for the rat. When synthetic pantothenic acid became available, Ration II was devised in which synthetic pantothenic acid and 0.25 per cent of

untreated 1:20 liver concentrate powder were substituted for the fullers' earth preparation. This eliminated the necessity of preparing a purified liver concentrate and gave a ration that could be easily reproduced. On both these rations the growth response is proportional to the amount of pyridoxine administered.

EXPERIMENTAL

Preparation of Butanol Extract of 1:20 Liver Concentrate Powder —1 kilo of 1:20 liver concentrate powder (Wilson Laboratories) was dissolved in 1 liter of water. 5 liters of butanol were added and the mixture shaken in a mechanical shaker for 1 hour. The layers were allowed to separate and the butanol layer removed. Four more similar extractions were made with 5 liters of butanol each time. In the last three extractions, 200 to 300 cc. of water were added each time to prevent the aqueous layer from becoming too thick to allow proper mixing and extraction. The butanol extracts were combined and concentrated under a vacuum. Water was added to aid in the removal of the last traces of butanol. The residue was taken up in 1 liter of water and allowed to stand overnight in the refrigerator. The insoluble material was removed by suction filtration and washed well with water.

Preparation of Fullers' Earth Filtrate of Butanol Extract of 1:20 Liver Concentrate Powder—The above butanol extract was diluted to 2 liters with water and acidified to pH 1.5 with hydrochloric acid. 50 gm. of English fullers' earth (A. Daigger and Company, Chicago) were added and the mixture shaken for $\frac{1}{2}$ hour. The fullers' earth was removed by suction filtration. The absorption was repeated four times. The fullers' earth fractions were combined and washed four times with 400 cc. of 0.1 N hydrochloric acid. These washings were combined and treated four times with 15 gm. of fullers' earth in the same manner as the main fraction. The main filtrate and the treated washings were combined, made just acid to litmus by the addition of sodium hydroxide, and then concentrated *in vacuo* to about 500 cc. The concentrate was filtered through Filter Cel and diluted to 1000 cc., so that 1 cc. of the concentrate was equivalent to 1 gm. of 1:20 liver concentrate powder.

Assay Procedure—Male rats 21 days old were placed on the depletion rations (Table I) which were fed *ad libitum*. In Ration I

the fullers' earth filtrate was administered as a supplement each day at a level equivalent to 0.4 gm. of 1:20 liver concentrate powder per day. In 2 to 3 weeks a definite plateau in the growth rate resulted.

Fig. 1 indicates the growth responses after the depletion period, at which time the rats were supplemented with either crystalline pyridoxine or a liver fraction. After the depletion period the

TABLE I

Composition of Depletion Rations

	Ration I	Ration II
	gm.	*gm.*
Sucrose	75	75
Casein (Labco)	18	18
Salts IV*	4	4
Corn oil†	3	3
	mg.	*mg.*
Thiamine	0 20	0 20
Riboflavin	0.30	0.30
Nicotinic acid	2.5	2.5
Choline	30 0	200.0
Pantothenic acid		0.50
1:20 liver concentrate powder‡		250 0
Haliver oil per wk.	2 drops	2 drops

* Phillips and Hart's salt mixture (12) with the $MnSO_4 \cdot 4H_2O$ increased from 0.7 to 10 gm. per kilo of salt mixture.

† Corn oil is added to the remainder of the basal ration only in amounts sufficient to last about 1 week.

‡ The 1:20 liver concentrate powder is incorporated into the ration by dissolving 10 gm. in 40 cc. of water and spreading with the aid of a pipette the proper volume together with aqueous solutions of the vitamins over a thin layer of casein. The casein is then dried at 30–40° and ground finely.

control rats grew less than 2 gm. per week for the 5 week assay period, while those on 2, 5, and 10 γ of pyridoxine per day grew 9, 14, and 19 gm. per week respectively. Similarly on the liver fractions the growth responses were 9.5, 12, 16, and 24 gm. per week on the levels of 0.025, 0.05, 0.1, and 0.2 gm. per day respectively. These results demonstrate that the growth response is correlated with the amount of vitamin B_6 administered.

In Ration II the liver concentrate powder and the pantothenic
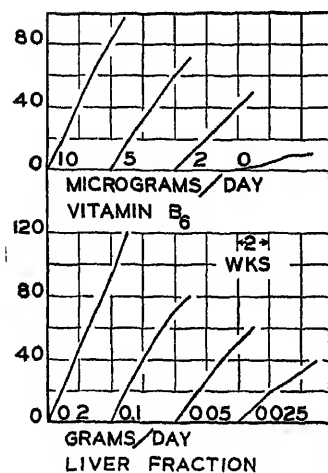
FIG. 1.
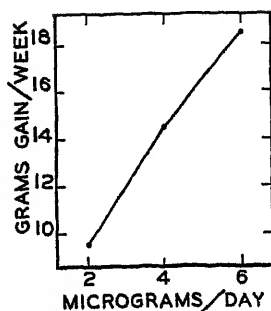
FIG. 2.

FIG. 1. Growth responses of rats on Ration I to vitamin B₆ and Wilson's liver fraction No. 41258.

FIG. 2. A typical calibration curve of vitamin B₆ hydrochloride against the average gain per week.

TABLE II

Growth Responses on Ration II with Various Supplements

Supplement	Amount per day	Average gain per wk.	Vitamin B₆ HCl* content
	γ	gm.	γ per gm.
Vitamin B₆ hydrochloride	2	8	
" " "	4	14	
" " " .. .	6	19	
" " "	10	28	
	gm.		
1:20 liver concentrate powder . . .	0.05	9	45
1:20 " " " . .	0.10	16	
Liver Fraction B	0.05	7	25
" " ".	0.10	10	
Yeast...	0.05	11.6	55
"	0.10	17	
Riboflavin concentrate (Commercial Solvents Corporation) .	0.33	17	15
Dried grass . . .	0.5	13.5	8

* To convert to vitamin B₆ content multiply by 0.82.

acid were incorporated directly in the basal rations. In 4 to 6 weeks growth stopped entirely or the rate of growth was very poor. As in the case of the other ration the supplements were administered at this point. Table II designates the growth response of rats on Ration II to graded amounts of pyridoxine and certain materials assayed. In Fig. 2 the average growth responses for the 5 week assay period are plotted against the level of pyridoxine hydrochloride. Each point represents the average of at least seven rats. The growth response shows a linear relationship to the level of pyridoxine. On the basis of this standardization the growth response to an unknown may be translated in terms of the amount of pyridoxine present in the material.

<div align="center">DISCUSSION</div>

In the preparation of the fullers' earth filtrate, a variable amount of the pantothenic acid content of the 1:20 liver concentrate powder may be lost. It would, therefore, be advisable to add synthetic pantothenic acid to Ration I to insure an adequate amount of this factor at all times. When sufficient pantothenic acid is present, rats will gain 28 to 30 gm. per week when supplemented with adequate amounts of pyridoxine, thus indicating the completeness of the ration. Since the rats stopped growing in 2 to 3 weeks, the ration is very low in vitamin B_6. Consequently, it would be a suitable ration for physiological studies on vitamin B_6 as well as for assay purposes.

It was felt that in certain cases a simple ration which could be easily reproduced was desirable. With this in mind Ration II was designed. Since pantothenic acid can now be supplied in crystalline form, the liver concentrate can be reduced to a level of 0.25 per cent in the ration. Although at this level the liver concentrate contributes about 10 γ of pyridoxine per 100 gm. of ration, the ration is still satisfactory for assay purposes. Ration II also contains a higher level of choline. Rats receiving 30 mg. of choline per 100 gm. of ration developed fatty livers. On autopsy the livers had a speckled appearance. Fat analysis of these livers gave values of about 6 per cent fat on the fresh basis. When the choline content of the ration was increased to 200 mg. per 100 gm. of ration, the fatty liver condition disappeared and the fat content was within normal limits.

The fact that 0.25 per cent of 1:20 liver concentrate powder may not supply optimum amounts of the unknown factors does not alter the usefulness of this simplified ration as an assay procedure. When adequate amounts of pyridoxine are added to the ration, the rats gain 25 to 28 gm. per week. The levels of 2, 4, and 6 γ of pyridoxine were found to be suitable suboptimal levels on which to establish a growth response relationship. Two groups of rats, one raised in the departmental stock colony and the other from a commercial source, responded quite similarly to this technique. However, a standardization should be established for each particular group of rats used.

On our rations no dermatitis has been noted during the depletion period. However, preliminary work indicates that if the casein level is increased to 30 per cent at the expense of the sucrose, the typical dermatitis results in 4 to 6 weeks. This may explain why some workers have not obtained dermatitis consistently and others have not been able to do so at all. Ration II contains 3 per cent corn oil which supplies adequate amounts of linoleic acid. Some rations of other laboratories contain no fat or a fat low in linoleic acid such as butter fat or hydrogenated vegetable oils (Kuhn and Wendt (13); Schneider et al. (10); Halliday and Evans (14); Dann and Subbarow (15)). Other rations contain an increased amount of protein (Dimick and Schreffler (7); Lepkovsky et al. (16)). Whether the higher levels of protein actually increase the vitamin B_6 requirement or are only related to the production of dermatitis remains to be studied.

SUMMARY

1. A biological assay method for pyridoxine is described in which either a fullers' earth filtrate of butanol extract of 1:20 liver concentrate powder is used as the source of unknown factors for the rat or 1:20 liver concentrate is used directly for this purpose.

2. The pyridoxine content of several materials is given.

BIBLIOGRAPHY

1. György, P., *Biochem J.*, **29**, 760 (1935).
2 Lunde, G., and Kringstad, H., *Biochem. J.*, **32**, 708 (1938).
3. Wilson, H. E. C , and Roy, G. K., *Indian J. Med. Research*, **25**, 879 (1938).

4 Schneider, H. A., Ascham, J. K., Platz, B. R., and Steenbock, H., *J. Nutrition,* **18,** 99 (1939).

5. Halliday, N., and Evans, H M., *J. Biol. Chem.,* **118,** 255 (1937).

6. Edgar, C E , El Sadr, M. M , and Macrae, T. F., *Biochem. J.,* **32,** 2200 (1938).

7. Dimick, M. K., and Schreffler, C. B , *J. Nutrition,* **17,** 23 (1939).

8 Reedman, E. J , Sampson, W. L., and Unna, K., *Proc. Soc. Exp. Biol. and Med.,* **43,** 112 (1940).

9. Birch, T. W , *J. Biol. Chem.,* **124, 775** (1938).

10 Schneider, H., Steenbock, H., and Platz, B. R., *J. Biol. Chem.,* **132,** 539 (1940).

11. Dann, W. J., *Proc Am. Soc. Biol. Chem ,* *J. Biol Chem.,* **128,** p. xviii (1939).

12. Phillips, P. H , and Hart, E. B., *J. Biol. Chem.,* **109,** 657 (1935).

13 Kuhn, R., and Wendt, G., *Z. physiol. Chem.,* **256,** 127 (1938).

14. Halliday, N., and Evans, H. M., *J. Nutrition,* **13,** 657 (1937).

15. Dann, W. J., and Subbarow, Y., *J. Nutrition,* **16,** 183 (1938).

16. Lepkovsky, S., Jukes, T. H , and Krause, M. E., *J. Biol. Chem.,* **115,** 557 (1936).

STUDIES ON THE PRESENCE OF NON-CARBON MON-OXIDE-COMBINING (INACTIVE) HEMOGLOBIN IN THE BLOOD OF NORMAL PERSONS

By ESTHER AMMUNDSEN

(From the Medical Department B of the University Clinic, Rigshospital, Copenhagen, Denmark)

In previous papers on this subject (1–3) the writer has reported studies showing, in contrast to previous views, that the blood of normal persons may contain a derivative of hemoglobin that is able to combine with CO only after reduction with sodium hyposulfite. In the following a report will be given of the results of 82 examinations carried out in 1937–38 on normal persons by a modification of the method of Van Slyke and Hiller (4, 5) for determination of methemoglobin.

Previously it was the prevailing view that the oxygen capacity (or the carbon monoxide capacity, which is equal to the oxygen capacity (6–8)) constitutes a measure of the total amount of hemoglobin in the blood except in certain pathological conditions (especially intoxications) associated with the presence of such derivatives of hemoglobin as sulfhemoglobin and methemoglobin, which are not able to combine with O_2 or CO.

In recent years, however, various authors (Weise (9), Heilmeyer and Sundermann (10), Bierring, Nielsen, and Nielsen (11), Price-Jones (12)) have reported some observations suggesting that under certain circumstances the circulating blood may contain derivatives of hemoglobin or hemoglobin-like substances that are not recorded by the direct gasometric methods, whereas their presence is ascertained through the control determinations, whether these be colorimetric (11, 12), spectrophotometric (9, 10), determinations of the iron content (9), or combinations of these methods. In several cases these authors found lower values for the hemoglobin content when it was estimated by the

oxygen capacity than when measured otherwise on whole blood, while this is claimed not to have been the case in determinations carried out on pure isolated hemoglobin. Some of the authors (9, 10), therefore, warn against application of the results obtained on purified hemoglobin solutions to whole blood, in which the conditions are considerably more complicated, and where Weise (9) at any rate thinks that inactive hemoglobin derivatives possibly may occur.

In normal blood Barkan and collaborators (13, 14) have found two hemoglobin-like substances, containing the so called "leicht abspaltbares Eisen," which are designated as pseudohemoglobin and pseudomethemoglobin. The latter is said to be unable to combine with O_2 or CO, but treatment with sodium hyposulfite converts it into a substance which can take up O_2 or CO.

Thus it may be said that recent investigations have raised a question about the correctness of the old view concerning the serviceability of the oxygen capacity as a measure for the total amount of hemoglobin in the normal blood.

The following studies were carried out by a modification of Van Slyke and Hiller's method for determination of methemoglobin. In this method the carbon monoxide-combining capacity of the blood is determined before and after reduction with $Na_2S_2O_4$; the increase in CO capacity caused by $Na_2S_2O_4$ is taken as a measure of the methemoglobin, since methemoglobin, which does not combine with CO, is reduced by hyposulfite to normal reduced hemoglobin, which combines quantitatively with CO. Since it appears possible that some hemoglobin derivatives, other than methemoglobin, may be measured by this method, we shall use the term "inactive hemoglobin" to designate the "methemoglobin" determined by the method of Van Slyke and Hiller.

<div align="center">EXPERIMENTAL</div>

The method as described by Van Slyke and Hiller is employed, but with a few modifications, the more essential of which are the following.

As suggested by Rappaport and Köck-Molnar (15), instead of water as a diluent a strong solution of urea (500 gm. in 1000 cc. of H_2O) is employed; it prevents precipitation of protein. From Table I it appears that the results are not influenced by this change.

TABLE I

Comparison between Analyses with Urea Solution and with Water for Dilution of Blood

The results are given in terms of volumes per cent of CO capacity of the blood

Blood No	Active Hb determined without $Na_2S_2O_4$ addition		Total Hb determined after $Na_2S_2O_4$ treatment	
	Urea solution	Water	Urea solution	Water
1	8.41	8.34		
2	17.64	17 48		
	17 44	17.71		
3			18.20	18.07
			18 05	

TABLE II

Variations Found in Subject E. A. (Female) Examined on Different Dates

Date	Total Hb	Active Hb	Inactive Hb calculated as difference between means for total and active
1937	*vol. per cent*	*vol. per cent*	*vol. per cent*
Feb. 3	16 26	14.43	1 88
	16 50	14.57	
" 5	16.43	15.43	0.97
	16 43	15.50	
Mar. 1	18 32	17.49	0.84
	18.16	17 32	
June 16	18 13	16.58	1.36
	18 05	16 87	
July 26	17.89	17.83	0
	17.59	17 76	
" 29	16.96	16 51	0.51
		16 38	
Aug. 20	16 53	16 51	0
	16.58	16.38	
Sept. 25	16.99	17 31	0
Oct. 12	15.46	15.04	0.42
1938	15.57	15 15	
Oct. 10	17.12	16 90	0
	17.45		

The shaking time, given by Van Slyke and Hiller as 1.5 minutes, is prolonged to 3 minutes, as this gives more security.

Instead of air-free 1 N NaOH a 5 N solution is employed for the

CO_2 absorption, as the 5 N solution need not be kept air-free. This change was found also not to alter the results.

The blood was stabilized with potassium oxalate, and as a rule the analyses were carried out immediately after the blood sample

TABLE III

Results from Series of Normal Subjects

Subject	Sex	Date	Total Hb	Active Hb	Difference
			vol. per cent CO capacity	*vol. per cent CO capacity*	*vol. per cent CO capacity*
E. K.	M.	July 6, 1937	21.09	20.60	0.49
N. T.	F.	Feb. 7, 1937	18.95	17 98	0.97
J. E.	M.	" 25, 1937	22.95	22.55	0.40
E. H.	"	Mar. 3, 1937	19.01	18.82	0
A. N.	F.	" 16, 1937	20.80	19.30	1.50
D. N.	"	" 10, 1937	18.20	17 64	0.61
P. P.	M.	Sept. 9, 1937	20.90	20.27	0.63
E. A.	"	" 22, 1937	21.14	20.91	0
V. A.	F.	" 28, 1937	19.09	17.93	1.16
J. F.	M.	Oct. 20, 1937	19.30	18.16	1.14
V. P.	F.	" 25, 1937	20.19	19.78	0.41
?	?	June 23, 1938	20.48	20.55	0
Average	.		20.18	19.54	0.61
E. H.	F.	Feb 4, 1937	17.69	17.02	0.67
		" 9, 1937	18.08	17.59	0.49
M. K.	"	Oct. 15, 1937	20.01	18.30	1.71
		Nov. 10, 1937	18.49	18.53	0
M. F.	M.	Dec. 11, 1937	23.22	20.60	2 62
		Mar. 29, 1938	22.13	20.59	1.52
M. T.	"	Feb. 22, 1937	19.88	19.83	0
		Aug. 23, 1937	20.41	19.24	1.17
		Oct. 16, 1937	20.64	19.98	0.66
		Mar. 22, 1938	19.25	18.27	0.98
M. P.	F.	Feb. 3, 1937	16.97	16.81	0
		" 5, 1937	16 50	16.59	0
		Mar. 3, 1937	17.10	16.83	0
		Sept 1, 1937	18.32	17.67	0.65

was taken, but in a few cases later, though always within 24 hours. The CO capacity was found to undergo no change within this length of time when the blood was kept in the ice box, whereas the active CO capacity was decreased when the blood was left standing for a longer time.

Results

Normal Subjects—Table II gives the results of duplicate analyses performed on blood samples drawn on ten different dates from one
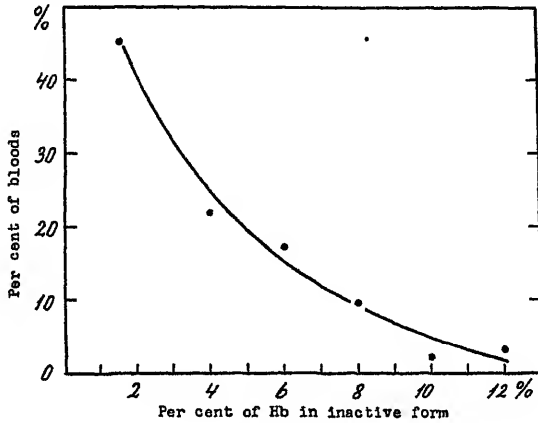
FIG. 1. Proportion of inactive hemoglobin found in 82 examinations of blood from 53 normal subjects. The abscissae indicate percentages of blood specimens examined, not percentages of subjects.
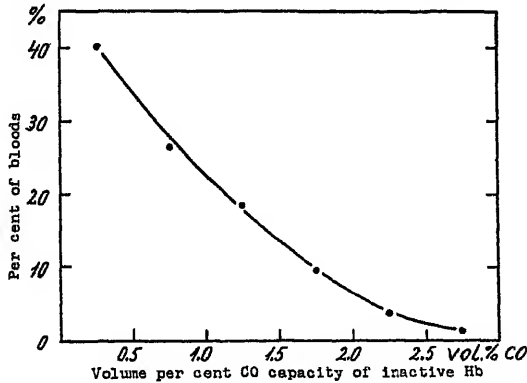
FIG. 2. Concentrations of inactive hemoglobin, in terms of volumes per cent of CO capacity, found in 82 analyses of blood from 53 normal subjects The abscissae indicate percentages of the blood specimens examined.

subject. These data serve to show the consistency of the analytical results indicated by the agreement between duplicates, and to show the variations which can occur in the blood of a normal subject.

Table III gives some of the results obtained from 52 other normal subjects, some of whom were examined two or four times, so that the total number of examinations was 72.   Each value for CO capacity in Table III is the average of duplicate analyses.

The results with normal subjects are summarized statistically in Figs. 1 and 2.   Values for inactive hemoglobin indicated by less than 0.5 volume per cent of CO capacity may be considered as within the limit of possible error.

Pathological Subjects—Single or repeated examinations have been carried out on the blood of thirty-five hospital patients with a variety of infectious and metabolic diseases, including polycythemia, myelogenous leucemia, pernicious anemia, aplastic anemia, disseminating sclerosis, and various infections.   The inactive hemoglobin values showed in none of these conditions variations exceeding those found in normal subjects.   Hence the results will not be reported in detail in this paper.

DISCUSSION

The analyses appear to show that a variable part of the hemoglobin circulating in the blood stream is present in a form able to combine with CO only after reduction with sodium hyposulfite. It has not been possible to demonstrate any cause of the variable amount of this abnormal hemoglobin in the blood.

According to previous views, methemoglobin is the only known reducible derivative of hemoglobin that may occur in the blood (under pathological conditions), and it was natural therefore to think that the substance demonstrated in the present work was methemoglobin.   It has not been practicable, however, with certainty to decide this question, as the substance could not be identified by ordinary spectroscopic examination.   This was really not surprising, as it was found experimentally (3) that when hemoglobin was converted in part to methemoglobin by addition of potassium ferricyanide it was not possible with the spectroscopes employed to see the spectrum of methemoglobin if the specimen contained less than 15 to 20 per cent methemoglobin.   (In some cases of intoxication, 15 to 25 per cent inactive hemoglobin was demonstrated by Van Slyke and Hiller's method.   In these cases there was a distinct methemoglobin spectrum with an absorption band at 630 $\mu\mu$ (3).)

According to Conant, Scott, and Douglass (16), hematin is included in the total hemoglobin, not in the active, in analyses by Van Slyke and Hiller's method. Accordingly, the substance found in the present analyses might possibly be hematin.

That it might be sulfhemoglobin does not seem probable, as investigations reported so far have shown it to be non-reducible.

So we have to assume that the demonstrated difference between the total and active hemoglobin contents of the blood is due to the occasional occurrence of reducible hemoglobin derivatives, the composition of which has not been established. Possibly it may be methemoglobin (or hematin); or possibly this substance may be identical with the "pseudomethemoglobin" found by Barkan recently.

SUMMARY

Determinations of methemoglobin in the blood of 53 normal subjects have been made by the method of Van Slyke and Hiller (8), in which the increase in carbon monoxide capacity caused by treating blood with $Na_2S_2O_4$ is used as a measure of the methemoglobin. The method was modified by using concentrated urea solution instead of water to dilute the blood. The "methemoglobin" determined by this method is called "inactive hemoglobin," because its identity appears uncertain.

In 60 per cent of the normal blood specimens examined the inactive hemoglobin found was equivalent to less than 0.5 volume per cent of CO capacity, which may be within the limit of error. In 40 per cent, however, inactive hemoglobin equivalent to from 0.5 to 2.5 volumes per cent of CO capacity was found The same subject examined on different days might show sometimes more, sometimes less, inactive hemoglobin than corresponds to 0.5 volume per cent of CO capacity.

A series of thirty-five hospital patients with various blood and other diseases gave similar figures. No disease was found in which high inactive hemoglobin was characteristic

Inactive hemoglobin is so frequently a significant part of the total hemoglobin that determinations of oxygen or carbon monoxide capacities do not appear to be accurate measures of the total hemoglobin content of blood.

BIBLIOGRAPHY

1. Ammundsen, E., *Ugesk Laeger*, **99**, 908 (1937); *Science*, **90**, 372 (1939).
2. Ammundsen, E., and Trier, M., *Acta med Scand.*, **101**, 451 (1939).
3. Ammundsen, E., Om ikke-kuliltebindende Haemoglobin i Blodet under normale og patologiske Forhold, Copenhagen (1939).
4. Van Slyke, D. D., *J. Biol. Chem*, **66**, 409 (1925).
5. Van Slyke, D. D , and Hiller, A , in Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, 349 (1932).
6 Sendroy, J , Jr , *J. Biol. Chem.*, **91**, 307 (1931).
7. Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., *J. Biol. Chem.*, **105**, 597 (1934).
8. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, **78**, 807 (1928).
9. Weise, W , *Biochem. Z.*, **293**, 65 (1937).
10. Heilmeyer, L., and Sundermann, A., *Deutsch. Arch. klin. Med.*, **178**, 397 (1936).
11. Bierring, E , Nielsen, A., and Nielsen, E., *Acta med. Scand.*, suppl. 78, 174 (1936)
12. Price-Jones, C., *J. Path. and Bact.*, **34**, 799 (1931).
13. Barkan, G., *Z physiol. Chem.*, **210**, 1 (1933)
14. Barkan, G., and Schales, C , *Z. physiol. Chem.*, **248**, 96 (1937); **253**, 83 (1937).
15. Rappaport, F , and Kóck-Molnar, K., *J. Biol. Chem.*, **104**, 29 (1934).
16. Conant, J. B., Scott, N D., and Douglass, W. F., *J. Biol. Chem.*, **76**, 223 (1928).

THE CHOLINE CONTENT OF RATS ON VARIOUS CHOLINE-FREE DIETS*

By H. P. JACOBI, C. A. BAUMANN, AND W. J. MEEK

(From the Department of Biochemistry, College of Agriculture, and the Department of Physiology, Medical School, University of Wisconsin, Madison)

Choline synthesis in the rat is intimated by the fact that methionine can replace choline in preventing fatty livers (1), and by the observation that labeled methyl groups can be transferred from methionine to choline (2). However, the development of fatty livers (3) and of hemorrhagic kidneys (4) and the restricted growth (5, 6) in rats on low choline diets all suggest that preformed choline must be supplied in the diet for optimal physiological performance in this species. Choline appears to be an indispensable constituent of the diet for the chicken (7, 8). It is therefore desirable to have information on the choline content of the tissues of rats maintained on diets low in choline. This was obtained by means of a direct analytical technique.

Methods

Reactions which have been used for the determination of choline include its oxidation to trimethylamine (9), the formation of an insoluble complex with iodine (10, 11), the isolation of the chloroplatinate or chloroaurate (12, 13), and the precipitation of choline as the reineckate (14). In the latter method choline reineckate is separated from other reineckates by virtue of its insolubility in water and ethyl alcohol. When dissolved in acetone, it forms a red solution which can be measured colorimetrically. A method based on this reaction was studied in detail,

either the Bausch and Lomb spectrophotometer or the Evelyn photoelectric colorimeter being used to measure color intensity.

Chemical Determination of Choline—Approximately 1 gm. of the fresh tissue was weighed in a tared evaporating dish and 10 cc. of 1:1 alcohol-ether mixture added plus sufficient sea sand to cover the tip of a spatula. The tissue was then ground to a pulp with a pestle, and the pestle washed off several times with 1:1 alcohol-ether mixture. The volume at this point was about 20 cc. The dish was then covered with a watch-glass, and the mixture boiled gently for 3 minutes. The solution was cooled, filtered, and the filtrate evaporated to near dryness on a steam bath. The concentrate was saponified for 2 hours at 80° with 15 cc. of saturated $Ba(OH)_2$ and then neutralized to phenolphthalein with acetic acid. The insoluble material was filtered onto an asbestos pad and 5 cc. of 2 per cent ammonium reineckate in methanol added to the filtrate. The mixture was allowed to stand in the cold for 12 hours to insure complete precipitation.

The insoluble reineckate was then filtered with suction onto an asbestos pad supported by a Gooch button in the bottom of a short stemmed funnel, and washed with cold 95 per cent alcohol until the washings were colorless. The residue was then dissolved off the pad with acetone, and the pad washed with more acetone until colorless. The combined acetone solutions, 8 to 10 cc., were then filtered through Whatman No. 40 filter paper into an Evelyn colorimeter tube and the color intensity measured in an Evelyn photoelectric colorimeter, with a filter which transmits light at 520 mμ. Finally the volume of the solution was measured with a burette.

The constant for the colorimeter was obtained by averaging the results of forty-one determinations made over a period of 16 months on five standard solutions. $K = 747$ (15), where

$$K = \frac{\text{micrograms choline}}{\text{volume of solution} \times (2 - (\log \text{galvanometer reading}))}$$

The maximum deviation from this value was 8 per cent, the usual deviation, 2 per cent or less. The absorption of light was found to be a linear function of concentration over the range of concentrations studied, 0.5 mg. to 9 mg. One constant, therefore, was applicable to all determinations made in this range. The ac-

curacy was greatest when relatively large amounts of choline were determined; the method did not yield reproducible results for amounts of choline less than 0.5 mg.

The reagents used in the above procedure were selected for the following reasons. The ammonium reineckate was added in methanol rather than in water (14) because of its greater solubility in methanol. The addition of the requisite excess of reineckate was thus achieved without the introduction of inconveniently large volumes. Precipitation was as complete in aqueous methanol as in water. Choline could be cleaved quan-
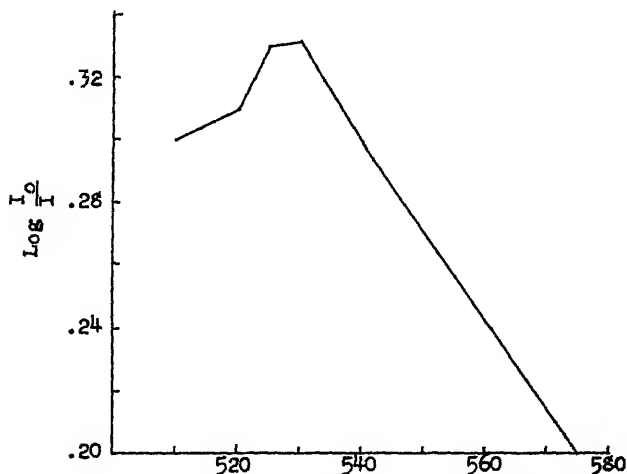
FIG. 1. The absorption spectrum (mμ) of choline reineckate in acetone. 1 cc. = 0.575 mg. of choline, viewed through 1 cm.

titatively from egg lecithin when heated with saturated Ba(OH)$_2$ for 1 hour at 60°. Thus, a purified preparation (16) with a P content of 3.92 per cent (theoretical, 3.99 per cent) was found to have a choline content of 16.9 per cent (16.50 to 17.56 per cent). The theoretical choline content was 17.92 per cent. For routine determinations, hydrolysis was effected by heating for 2 hours at 80° because of the possibility that choline complexes in tissue other than lecithin might be more resistant to hydrolysis. Choline was found to be stable under the latter conditions. Acetic acid was used to neutralize the Ba(OH)$_2$ after hydrolysis, because

barium acetate did not interfere with the subsequent precipitation
of choline reineckate. HCl, HNO_3, or H_2SO_4 yielded variable
results.

When the spectrophotometer was used to measure color in-
tensity, the maximum absorption of choline reineckate in acetone
was between 520 and 530 mμ (Fig. 1). The intensity of absorp-
tion at 525 mμ was expressed according to the formula $E = 1/cd$
log I_0/I, when E = extinction coefficient, c = concentration,
d = depth of solution, and I_0/I = ratio of the intensity of the
incident light to the intensity of the transmitted light. At a
depth of 1 cm. and for a concentration of 1 mg. of choline as choline
reineckate per cc. the extinction coefficient was 0.572. This was
the average of fourteen determinations made on two standard
solutions containing 1.5 or 3.0 mg. of choline per cc. The values
for the extinction coefficient varied from 0.511 to 0.669.

EXPERIMENTAL

The basal choline-free ration consisted of steamed egg white
18, corn oil 5, Wesson (17) salt mixture 4, cerelose 73. It was
supplemented with 20 γ of riboflavin and 10 γ of thiamine dis-
solved in N/28 acetic acid, with 10 γ of carotene and 5 γ of cal-
ciferol dissolved in partially hydrogenated coconut oil, and with
4 drops of choline-free rice bran concentrate[1] per rat per day.
An analysis of 100 gm. of the basal ration failed to reveal any
choline.

The choline-free rice bran concentrate was prepared as follows:
a measured volume of concentrate[1] was diluted with an equal
volume of water and 1 gm. of ammonium reineckate in methanol
added for every 30 cc. of concentrate. The mixture was allowed
to stand in the cold for 12 hours and the insoluble reineckate
filtered off and washed with cold water. The filtrate was then
concentrated *in vacuo* at a temperature not exceeding 60° until
most of the methyl alcohol had been driven off. 3.5 cc. of pyridine
were then added for every gm. of ammonium reineckate originally
used. The insoluble precipitate of pyridine reineckate was fil-
tered off after standing for several hours in the cold, the excess
pyridine removed *in vacuo*, and the choline-free concentrate
finally reduced to the original volume.

[1] Vitab Products, Inc., Emeryville, California.

For a study of the choline content of rats on various diets, twenty-six animals weighing 38 to 55 gm. were divided into eight groups comparable as to age, sex, and weight. Group 1, four rats, was killed for analysis at the beginning of the experiment. Group 2, seven rats, received the choline-free diet. Group 3, three rats, received the choline-free diet to which 35 per cent of hydrogenated vegetable oil[2] had been added at the expense of the cerelose. Group 4, two rats, received the choline-free diet supplemented with 7 mg. of choline per rat per day. Group 5, three rats, received a diet in which water and alcohol-extracted casein (18) replaced the egg white of the choline-free diet. No choline could be detected in 18 gm. of the casein. Group 6, two rats, received the same diet as Group 5 plus 0.5 per cent cystine. Group 7, three rats, received the same diet as Group 5 with the casein content increased to 40 per cent at the expense of the cerelose. Group 8, two rats, received a standard stock ration.[3]

The animals were kept in individual cages on screens, and were fed *ad libitum*. When the animals of Groups 2 to 8 had increased their weights at least 3-fold, they were killed, and the kidneys, liver, and brain removed and analyzed individually for choline. The remainder of the rat, designated as "carcass," was minced in a meat grinder, and extracted twice with 500 cc. portions of a 1:1 alcohol-ether mixture for 1 hour on a steam bath. 20 cc. aliquots of the combined filtrates were then analyzed for choline by the usual procedure.

Results

The animals on the various diets grew at a slow but continuous rate, the average weekly gain being 10 gm. Failure to obtain normal growth was apparently due to a deficiency of essential growth factors in the choline-free rice bran extract. The addition of choline to the choline-free diet failed to increase the rate of growth.

The results of the analyses clearly indicated that the rats had synthesized choline while on the choline-free diet. At the beginning of the experiment the choline content of the rats ranged from 38 to 51 mg., average 43 mg. (Table I, Group 1). After 8

[2] Primex.
[3] H. Steenbock, unpublished data.

to 18 weeks on the choline-free diet the content of the animals ranged from 118 to 209 mg., average 151 mg. (Group 2). The increase in choline therefore was 108 mg. As much as 76 mg. of choline was synthesized over an 8 week period by one animal.

TABLE I

Choline Content of Rats on Various Choline-Free Diets

Group No.	Diet	Wks. on diet	Weight of rat	Total choline				
				Kidney	Liver	Brain	Carcass	Total
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Stock		38	0.9	3.7	4.3	29	37.9
			40	0.7	3.9	3.4	31	39.0
			49	1.2	3.2	3.5	43	50.9
			50	0.9	3 3	3 7	37	44.9
2	Choline-free	8.1	138	2.0	7 5	4.0	105	118.5
		9 4	140	2.4	7.5	3.9	109	122.8
		12.6	178	2.9	15.0	5.0	117	139.9
		15.0	184	4.8	12.1	5.2	160	182.1
		15 0	192	2.6	17.5	5.2	184	209.3
		16.4	167	2.0	10.4	4.1	139	155.5
		17.6	164	2.8	12.9	4.6	106	126.3
3	High fat, choline-free	16.3	225	2.2	12.7	3.4	111	129.3
		16.3	230	2.9	17.9	5.1	135	160.9
		16 3	230	2.9	18.7	4.9	108	134.5
4	Choline-free + choline	16.2	220	2.5	13.5	4.8	179	199.8
		16.2	225	2.2	12.8	4.2	144	163.2
5	Casein	15.0	168	2.6	14.0	5.0	128	149.6
		15.0	170	2.3	13.3	4.7	134	154.3
		15.0	214	4.5	17.5	3.0	140	165.0
6	" + 0.5% cystine	15.0	198	2.3	13.1	5.6	142	163.0
		15 0	205	3.7	16.9	5.3	169	194.9
7	High casein	15.0	120	1.5	11.6	4.1	88	105.2
		15.0	196	2.4	17.5	5.2	145	170.1
		15.0	258	2 5	27.6	5.7	191	226.8
8	Stock	3.7	165	3.4	16.5	4.9	114	138.8
		4.9	220	3.0	19.2	5 2	147	174.4

The consumption of the basal ration during the experiment was 500 to 1500 gm. per rat, depending on the length of the experimental period. 100 gm. of ration contained less choline than could be detected chemically. Assuming that 0.1 mg. of choline was present per 100 gm., the maximum amount which could have

been consumed by any one rat during the experimental period was 15 × 0.1 or 1.5 mg., a negligible amount compared to the 108 mg. average increase observed per animal.

This increase in choline content was general throughout the body. The increase in any one tissue was approximately proportional to the increase in weight and the increase in choline content of the entire animal also paralleled the increase in weight, roughly 0.8 to 0.9 mg. of choline being present per gm. of rat whether choline was supplied in the diet or not. Animals on the choline-free diet (Group 2) contained approximately the same amount of choline as rats of similar weight raised on the stock diet (Group 8). The addition of 35 per cent fat (Group 3) to the choline-free diet appeared to reduce the choline content of the rats somewhat. The choline content per gm. of rat on the high fat diet ranged from 0.58 to 0.70 mg., average 0.62 mg. The average for the rats on the other diets (Groups 2, and 4 to 8) was 0.86 mg. of choline per gm. of rat, ranging from 0.73 to 1.1 mg. The other dietary variations employed, namely high choline (Group 4), replacement of egg white by casein (Group 5), high cystine (Group 6), and high casein (Group 7), did not alter appreciably the choline content of the rats. The experiments as performed did not indicate whether choline was synthesized by the tissues themselves or by the bacteria of the gastrointestinal tract. If the latter were true, choline might be expected in the feces. However, no choline could be detected in a 7 day specimen of the feces of two rats on a choline-free diet.

Biological Assay

The question remained whether the increase in choline as measured by the reineckate method represented only choline, or whether other substances in rat tissue yielded alcohol-insoluble, acetone-soluble reineckates. In other words, the specificity of the method had still to be demonstrated. This was done by comparing our chemical results with those obtained with a highly specific biological method; viz., the contraction of rectus abdominis muscle of the frog in the presence of acetylcholine and eserine.

The procedure employed was essentially that of Chang and Gaddum (19). The muscle was removed from the frog and allowed to bathe for 10 minutes in a Locke's solution containing

1 mg. of eserine salicylate in 100 cc. and agitated with a stream of
air.   The solution was prepared as follows (20): 38 cc. of water
were added to 100 cc. of stock solution containing 0.9 per cent
NaCl, 0.016 per cent $CaCl_2$, 0.0207 per cent KCl, 0.022 per cent
$NaHCO_3$, and 0.1 per cent glucose.   The resulting mixture had
the correct osmotic pressure for the tissues of cold blooded animals.

The muscle was then mounted in a bath consisting of an upright
tube 8 cm. long and 2.5 cm. in diameter.   The lower end of the
tube was fitted with a rubber stopper bearing a stop-cock, a
capillary tube, and a small wire hook.   The stop-cock was used
for draining the bath, the capillary for passing air into the bath,
and the hook for attaching one end of the muscle.   The other
end of the muscle was attached by an S-hook to a silk string which
ran over a pulley to a light lever, recording contractions on a
slowly revolving kymograph.   The test contractions were elicited
by solutions of acetylcholine which were placed in the bath, im-
mersing the muscle for a standard period of 6 minutes.   The
amounts of acetylcholine ranged from 0.5 to 2.0 γ per 20 cc. of
Locke's solution.   0.2 cc. of 0.1 per cent escrine salicylate in
Locke's solution was added to each of the 20 cc. aliquots which
bathed the muscle.   Consistent results were obtained only when
precautions were taken to overcome the friction of the lever on
the sooted drum.   This was accomplished by gently tapping
the rubber stopper of the bath with a pencil.

When the contraction period (6 minutes) was over, the solution
was drained out of the bath, and the muscle washed three times
over a 5 minute period with Locke's solution containing escrine.
Complete relaxation was obtained, and the muscle was then ready
for contraction in the presence of some other concentration of
acetylcholine, or of a sample to be assayed.   Under proper con-
ditions of washing and aeration, muscle samples retained their
sensitivity for several hours.   The assays of unknowns were
always preceded and followed by the measurement of contractions
in the presence of at least two known concentrations of acetyl-
choline.   Identical results were obtained with commercial acetyl-
choline or with choline chloride which had been acetylated by the
method of Abderhalden and Paffrath (21).

For the comparison of chemical and biological methods two
fractions from rat tissues were employed: (a) the crude alcohol-

ether extract of the tissues, and (b) the precipitated choline reineckate fraction. The former was prepared for analysis by a modification of the technique of Fletcher, Best, and Solandt (22). A 20 cc. aliquot was pipetted into a 250 cc. round bottom flask and evaporated to near dryness on a steam bath. 40 cc. of 18 per cent HCl were added and the mixture refluxed for 1 hour. The HCl was then removed by evaporating to near dryness *in vacuo* and 30 cc. of water added. The solution was extracted with twice the volume of ether to remove fatty acids and unhydrolyzed

TABLE II

Determination of Choline Content of Rat Tissue by Chemical and Biological Methods of Assay

Determination No.		Acetylation of	Choline	
			Chemical	Bioassay
			γ	γ
1	Duplicate	Alcohol-ether extract	2212	2050
2	aliquots		2125	2050
3	" "	" "	2140	2130
4			2108	2360
5	" "	Decomposed reineckates	3759	3521
6			3742	3675
7		" "	4900	4500
			mg.	mg.
8		" choline reineckate prepared from weighed samples of choline	15 8*	18.5
9		" "	45.1*	45 0
10		Weighed sample of choline	26.8*	27.2

* Weighed.

fat. The ether extract was washed twice with 20 cc. portions of water and the washings returned to the mother liquor. The combined solutions were then evaporated to dryness *in vacuo* at a temperature not exceeding 60°. Small portions of absolute alcohol were added and evaporated off to remove the last traces of water. The residual choline chloride was then acetylated (21) by adding 25 cc. of glacial acetic acid and 5 cc. of acetic anhydride to the residue and refluxing for 2 hours. The solution was evaporated to complete dryness at 60°, a small portion of

absolute alcohol added, and the evaporation repeated. The residue was transferred to a 100 cc. volumetric flask and made up to volume with Locke's solution, and assayed according to the above procedure.

For the biological assay of choline reineckate the following procedure was employed: An acetone solution of the reineckate was diluted with an equal volume of water and treated with twice the theoretical amount of silver sulfate dissolved in a minimum of water. The mixture was allowed to stand in the cold for 12 hours, and the insoluble silver reineckate filtered off through an asbestos pad and washed several times with a 1:1 acetone-water mixture. The calculated amount of $BaCl_2$ necessary to precipitate the excess silver sulfate was then added to the filtrate and the precipitated $BaSO_4$ and $AgCl$ filtered off. The filtrate was evaporated to complete dryness and acetylated as before.

The most pertinent results were obtained with choline fractions from rats which had increased their choline content while on a choline-free diet. The results (Table II) indicate a good agreement between the chemical and biological methods of assay, variations being within the limits of error of the latter method. In other words, "choline" as determined in rat tissue by the reineckate method consisted only of choline, and the increased choline content of the tissues represented a true biological synthesis.

DISCUSSION

The choline content of the tissues as determined by the reineckate method agrees satisfactorily with the results obtained by Best and coworkers (22) using a biological assay. Liver, kidney, and brain have an average total choline content of 2.3, 2.0, and 3.0 mg. of choline per gm. of tissue, respectively. The constancy of the amount of choline in any given tissue, despite dietary variations, indicates that the rat is able to catabolize choline rapidly as well as to synthesize it. The existence of a choline oxidase in tissues has been reported by Bernheim and Bernheim (23).

The work of du Vigneaud et al. (2) would suggest that methionine can function in methylating an unknown precursor to form choline. Inasmuch as all the diets used in the present study were adequate with respect to methionine, the source of the

methyl groups in the choline synthesized by the rats is probably accounted for.

In spite of the demonstrated ability of the rat to synthesize choline, the need for it in preventing fatty livers (3) and kidney degeneration (4) would suggest that the rat can synthesize choline only at a limited rate. This may not be great enough to satisfy the needs of the animal at all periods of the life span or under all dietary conditions. In other words choline might be regarded as a semidispensable constituent of the diet, roughly comparable in this respect to arginine.

SUMMARY

The choline content of rats on choline-free diets increased with the weight of the animals, thus indicating choline synthesis. As much as 76 mg. of choline was synthesized over an 8 week period by one animal. Choline synthesis was reduced somewhat on a diet high in fat, but other variations in the low choline diet, high protein, high cystine, and a change of protein, as well as the addition of choline, were without marked effect on the choline content of the tissues.

Details are given for the determination of choline by the reineckate method, and also by the biological method with contraction experiments with frog muscle. Results obtained by the two methods agree satisfactorily.

BIBLIOGRAPHY

1. Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, **126**, 117 (1938).
2. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).
3. Best, C. H., and Huntsman, M. E., *J. Physiol.*, **75**, 405 (1932).
4. Griffith, W. H , and Wade, N. J., *J. Biol Chem.*, **132**, 627 (1940).
5. Sure, B., *J. Nutrition*, **19**, 71 (1940).
6. McHenry, E. W., *Biochem. J.*, **31**, 1617 (1931).
7. Abbott, O. D., and DeMasters, C. U., *J. Nutrition*, **19**, 47 (1940).
8. Jukes, T. H., *J Biol Chem.*, **134**, 789 (1940).
9. Lintzel, W., and Monasterio, G., *Biochem. Z.*, **241**, 273 (1931).
10. Roman, W., *Biochem. Z.*, **219**, 218 (1930).
11. Erickson, B. N., Avrin, I., Teague, D M., and Williams, H. H., *J. Biol. Chem.*, **135**, 671 (1940).
12. Malengreau, F , and Prigent, G , *Z physiol Chem* , **77**, 107 (1912).
13 Kinoshita, T , *Chem Zentr* , **2**, 235 (1910).
14 Beattie, F. J. R., *Biochem J* , **30**, 1554 (1936)

15. Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).
16. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **72**, 587 (1927).
17. Wesson, L. G., *Science*, **75**, 339 (1932).
18. Quackenbush, F. W., Platz, B. R., and Steenbock, H., *J. Nutrition*, **17**, 115 (1939).
19. Chang, H. C., and Gaddum, J. H., *J. Physiol.*, **79**, 255 (1933).
20. Bunting, H., Meek, W. J., and Maaske, C. A., *Am. J. Physiol.*, **114**, 100 (1935).
21. Abderhalden, E., and Paffrath, H., *Arch. ges. Physiol.*, **207**, 228 (1925).
22. Fletcher, J. P., Best, C. H., and Solandt, O. M., *Biochem. J.*, **29**, 2278 (1935).
23. Bernheim, F., and Bernheim, M. L. C., *Am. J. Physiol.*, **121**, 55 (1938).

THE DISTRIBUTION OF BODY WATER IN SKELETAL MUSCLE AND LIVER IN NORMAL DOGS FOLLOWING INJECTIONS OF POTASSIUM SALTS*

By LILLIAN EICHELBERGER

(*From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago*)

This investigation of the changes in water and electrolyte distribution in skeletal muscle following an experimental increase in body potassium and total body water is a continuation of previous studies. Data will be presented on the volumes of extracellular and intracellular phases of skeletal muscle of normal dogs, both before and after increases in potassium and total body water produced by the intravenous injection of an isotonic solution containing potassium. These findings will be compared statistically with those obtained both previous to and following the intravenous injection of an isotonic solution of sodium chloride on normal animals serving as controls.

In addition, there will be included analyses of liver from a group of normal animals, both before and after the injection of the potassium salts. These data will be compared with those for the skeletal muscle for the purpose of determining whether injected potassium is concentrated in certain tissue cells. These findings will add more experimental evidence to the tentative conclusions of Fenn (1) that the liver has some special ability to absorb injected potassium.

EXPERIMENTAL

Normal dogs were used for all injections. All dogs were maintained under observation in metabolism cages for approximately 2 weeks and were in excellent physical condition at the

---

time of experimentation. Before an experiment was carried out, the dog was weighed and anesthetized with sodium barbital as in previous experiments (2). After about an hour, the dog was placed on an operating board, the bladder catheterized for the collection of urine during the entire experimental period, and a cannula introduced into the carotid artery for continuous blood pressure tracings. Blood was taken under oil with a cannula in the femoral artery, and one rectus abdominis muscle was removed for the initial analyses.

The salt solution, warmed to 38°, was then injected by gravity through the femoral vein. The safe maximum speed of injection for potassium ions of a concentration as used in these experiments was found to be 32 to 35 cc. per minute. A faster rate caused toxic effects on the heart. The injection required from 50 to 60 minutes, after which 30 minutes were allowed to elapse before a second sample of blood was withdrawn, and the rectus abdominis muscle on the opposite side removed for the final analyses. The peritoneal fluid was then removed as completely as possible, the last traces by means of a sponge. In the group of dogs in which the changes in the liver were considered, the initial sample of liver for analysis was taken by biopsy at least 3 weeks before the injection experiment was carried out. The final sample of liver was then taken following the removal of the second rectus abdominis muscle.

The study included two groups of experiments: (1) Five normal animals received intravenous injections of 170 cc. per kilo of body weight of an isotonic salt solution consisting of 129 mM of NaCl + 25 mM of $NaHCO_3$; (2) twenty normal animals received intravenous injections of 170 cc. per kilo of body weight of an isotonic salt solution consisting of 129 mM of NaCl + 25 mM of $KHCO_3$. In a number of experiments, animals were given intravenously 170 cc. per kilo of body weight of a solution consisting of 119 mM of NaCl + 35 mM of $KHCO_3$. However, potassium in these amounts was found to be too toxic for these experiments.

Chemical Methods

The chemical methods for blood and skeletal muscle analyses were the same as those used in previous studies (3, 4). The blood was defibrinated for whole blood analysis. In all cases, both

whole blood and serum were analyzed for water, sodium, potassium, and chloride. Cell volume was also determined. Liver and skeletal muscle were analyzed for fat, blood, water, sodium, potassium, and chloride. The determination of blood depended upon the colorimetric comparison of the amount of hemoglobin in the tissue with that of the whole blood taken as nearly simultaneously as possible with the tissue. The volume of blood circulating in liver tissue being very large, to avoid loss the piece cut at biopsy was dropped instantly into a weighing bottle. The tissue was then minced with scissors and dried to constant weight in an oven at 101°. After the dried tissue was extracted for neutral fat, it was transferred quantitatively to a special apparatus and pulverized.[1]

Preparation of the dried liver for analysis and the analytical methods were the same as reported for kidney tissue in a former paper (6).

For the calculation of the volumes of extra- and intracellular fluids in tissues, the method previously described (2) was used.

The amounts of extracellular phase (F) in gm. per kilo of muscle were calculated from the equation

$$(F) = \frac{(Cl)_M \times (H_2O)_s \times 1000}{1.04 \times (Cl)_s}$$

in which the subscripts M and s represent muscle and serum respectively. From the values for (F), the intracellular phase (C) per kilo of muscle was estimated by the equation $(C) = 1000 - (F)$.

Both the liver and muscle analyses are reported in terms of fat-free, blood-free tissue. Since the muscle analyses are corrected for blood as well as for fat, the control data on the muscle of normal dogs previously reported on a fat-free basis only could not be used here. Some error may occur as a result of these

---

[1] This apparatus made from stainless steel (Graeser, Ginsberg, and Friedemann (5)) consists of a base 7 1 cm. in diameter permanently attached to a round, solid piece of steel 2 5 cm. in diameter and 0 5 cm. high, which serves as an anvil upon which the dry tissue is crushed. The anvil is surrounded by a permanent steel cylinder 7.7 cm. in diameter × 6.7 cm. high. The removable cylinder, 2.6 cm. in diameter × 5.1 cm. high, rests upon the anvil. The plunger, which fits into the removable cylinder, is 2.5 cm. in diameter and 7.5 cm. high.

corrections, because it must be assumed that the concentration of the red cells in the tissue capillaries is the same as that in the arteries. It is interesting to note that the average amount of blood in the skeletal muscle as removed from the living dogs was very constant, 71 cc. per kilo of muscle, $\sigma \pm 11$; while the average amount in the liver as removed at biopsy was 197 cc. per kilo, $\sigma \pm 30$.

Results

Twenty-five experiments were performed, including five control animals and twenty animals receiving the intravenous injection of potassium.

Muscle Changes in Normal Dogs Following Isotonic Sodium Chloride Injections

Control Animals—The results, both preceding (initial) and following (final) the increase in total body water, are presented in detail in Table I for two representative experiments. In these normal animals the increase in total body water was produced by the intravenous injection of 170 cc. per kilo of body weight of a salt solution containing 25 mM of $NaHCO_3$ + 129 mM of NaCl per liter. This group of dogs was regarded as controls.

In all of these experiments there was little change in the blood pressure during and following the injection.

Muscle Phase Volumes—From such data as given in Table I, the extra- and intracellular phases and the intracellular water have been calculated by the method described above. These data with standard deviations are given in Table III. Before injection, the normal extracellular phase of 1 kilo of skeletal muscle amounted to 155 gm. with a standard deviation (σ) of ± 19.0 gm., and the intracellular phase amounted to 845 gm., ± 19.0 gm. Following the injection, the increase produced in the bulk of 1 kilo of muscle amounted to an average of 48 gm., ± 8 gm. per kilo of original muscle, which consisted of a 52 gm. increase in the extracellular phase and a -4 gm. decrease in the intracellular phase. Therefore, the experimental edema produced in a kilo of fat-free, blood-free skeletal muscle following an increase in total body water produced by the injection of isotonic sodium chloride solution was entirely accounted for by an increase in the extracellular phase of the skeletal muscle.

TABLE I

Control Animals. Changes in Blood and Muscle after Injection of Normal Isotonic Sodium Chloride Solutions

Solution, 129 mM of NaCl + 25 mM of NaHCO₃.

Dog 116; weight 10.6 kilos; 1800 cc. injected; peritoneal fluid 155 cc., urine 590 cc. Blood pressure, control, 120 mm.; after injection, 150 mm.

	pH	CO₂ (mM per l.)	H₂O (gm. per kg)	Cl (mM per kg.)	Na (mM per kg.)	K (mM per kg.)	Protein (gm. per kg.)	Blood (cc. per kg)	(F)
Serum. Initial	7.42	22.7	922.3	107.3	140.1	3.25	56.3		
Final	7.42	25.0	937.1	111.6	144.7	2.54	43.4		
	Sp. gr.	Hematocrit							
Blood.* Initial	1.051	47.1	791.7	84.97	121.2	5.17			
Final	1.044	40.1	818.0	91.36	126.0	4.03			
Peritoneal fluid per liter				119.1	151.8	2.58	21.5		
Urine per liter				128.5	131.4				
Muscle values corrected for total neutral fat									
Muscle. Initial			774.9	20.03	31.8	88.4		59	166
Final			784.9	25.63	39.7	87.5		55	207
Muscle values corrected for fat and blood									
Muscle. Initial			776.0	16.00	26.2	94.0			132
Final			784.0	21.80	34.7	92.6			167

Dog 117, weight 9.4 kilos, 1600 cc. injected; peritoneal fluid 190 cc., urine 277 cc. Blood pressure, control, 140 mm.; after injection, 140 mm.

	pH	CO₂ (mM per l.)	H₂O (gm. per kg.)	Cl (mM per kg.)	Na (mM per kg.)	K (mM per kg.)	Protein (gm. per kg.)	Blood (cc. per kg.)	(F)
Serum. Initial	7.30	24.2	920.2	110.3	144.9	4.40	60.7		
Final	7.35	24.6	945.0	113.1	146.8	3.41	38.8		
	Sp. gr.	Hematocrit							
Blood.* Initial	1.053	49.4	784.8	89.57	121.3	6.69			
Final	1.043	39.3	827.4	95.02	126.5	5.01			
Peritoneal fluid per liter				122.1	152.5	3.00	15.5		
Urine per liter				158.2	170.3				
Muscle values corrected for total neutral fat									
Muscle. Initial			771.9	26.20	37.56	87.7		105	210
Final			787.9	31.35	42.50	81.7		79	251
Muscle values corrected for fat and blood									
Muscle. Initial			776.0	18.81	27.80	97.9			151
Final			788.0	26.00	35.45	88.5			208

* The concentrations are expressed in mM per liter; the hematocrit, in per cent.

Muscle Changes in Normal Dogs Following Isotonic Salt Solution Containing Potassium

The results of two typical experiments, both preceding and following the increase in total body water and potassium are presented in Table II. The increase in total body water and potassium was produced by the intravenous injection of 170 cc. per kilo of body weight of a salt solution containing 25 mM of $KHCO_3$ + 129 mM of NaCl per liter.

In most of these experiments there was little change in the blood pressure, either during or following the injection. In three animals of this group, however, there were definite toxic heart reactions during the injection period.

Since the rate of excretion of potassium by the kidneys is generally rapid in dogs, the concentration retained in the organism in these experiments is shown by Table IV.

In the individual animals no correlation was found between the level of serum potassium and the potassium in skeletal muscle, which agrees with the findings of Darrow, Harrison, and Taffel (7). Although the present series of dogs was fed the same diet of alternate feedings of meat and dog chow,[2] the content of potassium in the skeletal muscle varied from 93.4 to 109.5 mM per kilo of fat-free, blood-free muscle, while the potassium in the serum did not vary proportionately with the muscle potassium.

Muscle Phase Volumes—From such data as given in Table II, the muscle phase volumes with standard deviations were calculated and are shown in Table III. After the injection, the increase produced in the bulk of 1 kilo of original muscle amounted to an average of 58 gm., σ ±23 gm., which consisted of a 33 gm., σ ±47, increase in the extracellular phase and a 25 gm., σ ±37, increase in the intracellular phase. Therefore, the experimental edema produced in 1 kilo of fat-free, blood-free skeletal muscle following the increase in total body water and total body potassium resulted from variable increases in both the extra- and intracellular phases.

Tissue Analyses—In order to show the changes in proportions of water, chloride, sodium, and potassium in muscle and liver following the increases in total body water and potassium, the

[2] Globe Dog Chow Blox.

TABLE II

Experimental Animals.   Changes in Blood and Muscle after Injection of Normal Isotonic Potassium Solutions

Solution, 129 mM of NaCl + 25 mM of KHCO₃.

Dog 108; weight 11 6 kilos; 1900 cc. injected; peritoneal fluid 188 cc.; urine 505 cc. Blood pressure, control, 150 mm.; final, 180 mm.

	pH	CO₂	H₂O	Cl	Na	K	Protein	Blood	(F)
		mM per l.	gm. per kg.	mM per kg.	mM per kg.	mM per kg.	gm. per kg.	cc. per kg.	
Serum. Initial	7.44	25.7	926.2	108.6	140.5	3 95	53 8		
Final	7.52	20.9	942.9	111.8	138.0	7 86	40.3		
	Sp. gr.	Hematocrit							
Blood.* Initial	1.051	42.0	776.2	90.3	122.6	6.51			
Final	1.046	38.2	824.5	94.7	122.5	9.35			
Peritoneal fluid per liter				121.7	144.0	7 65	17 9		
Urine per liter				168 0	159.5	37.6			
Muscle values corrected for total neutral fat									
Muscle. Initial			776.9	22.0	37.0	94.6		64	180
Final			791.8	25.3	37.5	90.6		66	205
Muscle values corrected for fat and blood									
Muscle. Initial			779.0	17.40	31.3	101.0			143
Final			792.0	20.45	31.6	96.7			166

Dog 107; weight 12 0 kilos; 2000 cc. injected; peritoneal fluid 110 cc.; urine 775 cc. Blood pressure, control, 180 mm.; final, 140 mm.

	pH	CO₂	H₂O	Cl	Na	K	Protein	Blood	(F)
		mM per l.	gm. per kg.	mM per kg.	mM per kg.	mM per kg.	gm. per kg.	cc. per kg.	
Serum. Initial	7.40	23.05	921.2	104.5	138.5	3 72	58.5	65	182
Final	7.48	20.00	938.4	112.0	140.0	5 00	43.7	53	210
	Sp. gr.	Hematocrit							
Blood.* Initial	1.050	43 9	804.5	86.2	119.3	5.05			
Final	1.046	39 6	821 8	92.0	120.0	5 57			
Peritoneal fluid per liter				118 5	143.0	5 20	17.8		
Urine per liter				122.0	114 5	30.0			
Muscle values corrected for total neutral fat									
Muscle. Initial			777.1	21 52	32.5	98.5		65	182
Final			796 2	25 95	36.6	90.9		53	210
Muscle values corrected for fat and blood									
Muscle. Initial			778 0	17.1	26.6	105.4			145
Final			797.0	22.3	32.0	96.0			180

* The concentrations are expressed in mM per liter; the hematocrit, in per cent.

TABLE III

Phase Volume Data from All Animals Grouped for Comparison

(F) = gm. of extracellular phase per kilo of muscle
(C) = " " intracellular " " " "
$M = (F) + (C)$
$\{H_2O\}_C$ = gm. of water per kilo of intracellular phase
σ = standard deviation
P = percentage probability
Δ = differences between means before and after injection
Δ_N = " " of normal animals receiving NaCl + NaHCO₃ injections and of normal animals receiving NaCl + KHCO₃ injections

	No. of animals	M			(F)			(C)			{H₂O}$_C$		
		Mean	σ	P	Mean	σ	P	Mean	σ	P	Mean	σ	P
		gm.		per cent	gm.		per cent	gm.		per cent	gm.		per cent
Control animals receiving 25 mM NaHCO₃ + 129 mM NaCl*													
Before injection	5	1000	±8		155	±19		845	±19		741	±4	
After injection	5	1048	±8		207	±18		841	±17		741	±6	
Δ		+48	±48	0 00	+52	±26	4.6	−4	±26	88	0	±7	100
Experimental animals receiving 25 mM KHCO₃ + 129 mM NaCl*													
Before injection	12	1000	±23		150	±27		850	±27		736	±8	
After injection	12	1058	±23		183	±39		875	±24		743	±10	
Δ		+58	±23	2 5	+33	±47	46	+25	±37	50	+7	±13	59
Δ$_N$		+10	±24	67	+24	±50	63	+34	±29	25	+2	±9	83

* All values are expressed per kilo of fat-free, blood-free muscle.

tissue analyses are expressed per 100 gm. of fat-free, blood-free solids and are summarized in Table V. It already has been pointed out (2) that the total water content of the tissue and consequently the total tissue solids are very constant if expressed in terms of fat-free tissue. These solids represent chiefly intracellular proteins and other intracellular constituents, and may be taken as a measure of the relative mass of tissue cells. When tissues swell, by increase in the extracellular fluid, the added fluid contains only about 1 per cent of solids, which is not sufficient to affect the total solids of the original tissue significantly.

TABLE IV

Potassium Intake, Excretion, and Retention of Twelve Dogs

Solution injected, 25 mM of $KHCO_3$ + 129 mM of NaCl.

Dog No.	Weight	Injection	K injected	K excreted	K retained	K retained per kilo*	Urine
	kg.	cc	mM	mM	mM	mM	cc.
103	12.2	2000	50.0	14.20	35 8	2 93	410
106	15.0	2300	57 5	18.78	38.7	2.58	637
107	12.6	2000	50.0	23 25	26.8	2.13	775
108	11.6	1900	47.5	15.30	32.2	2.78	505
109	8.9	1400	35.0	12 75	23 3	2.62	385
110	13 0	2000	50 0	21 50	29.5	2 27	520
111	11 0	1800	45 0	17.45	28 6	2.60	555
112	12.0	2000	50 0	12.48	37.5	3 13	450
113	13.0	2200	55.0	25 18	29.8	2.30	516
114	14.0	2200	55.0	24 00	31 0	2.21	440
DL$_6$	11.7	1600	40.0	17.20	22.8	1.94	330
DL$_7$	12.0	2000	50.0	20 75	29.2	2.44	455

* Average K retained = 2.50 mM per kilo.

By relating concentrations of water, chloride, sodium, and potassium to the total solids of muscle and liver (Table V), and treating the results statistically, it is possible to determine whether there has been any significant increase in the amounts of these substances in a unit mass of the original tissue. If there has been no such increase, no further analysis of the data is necessary. If, however, a significant increase has occurred, it is essential to determine whether the increase is in the intracellular phase or whether it is in the added extracellular phase.

Table V shows that no significant changes occurred in the skeletal muscle in this series of experiments. In the liver, how-

TABLE V

Summary of Analyses of Muscle and Liver of Normal Dogs before and after Increase in Total Body Water and Potassium

Δ = differences between means before and after injection.

Tissue values are given per 100 gm. of fat-free, blood-free solids.

	No. of animals	H2O (intracellular)			Cl			Na			K		
		Mean	σ	P	Mean	σ	P	Mean	σ	P	Mean	σ	P
		gm.		per cent	mM		per cent	mM		per cent	mM		per cent
Muscle													
Before injection	15	277	±15		8.18	±1.24		12 4	±2.83		44.4	±2.2	
After injection.. .	15	288	±15		10.41	±1.92		14 7	±2.92		46.0	±1.9	
Δ..... ·		+11	±23	62	+2.23	±2 29	32	+2.3	±4.07	57	+1.6	±2.9	56
Liver													
Before injection ·· ·	8	263	±9		9.52	±1.96					31.4	±2.4	
After injection .. ·	8	312	±18		11.05	±1.92					38 7	±1.7	
Δ... ··········		+49	±20	4.0	+1.53	±2.74	58				+7.3	±2.9	1.5

ever, both water and potassium concentrations were significantly increased. That the increase in water represents a swelling of the intracellular phase is demonstrated in Table VI. Assuming that all of the potassium in the plasma is diffusible, the maximum amount of potassium in the extracellular phase was calculated to be 0.6 mM per 100 gm. of fat-free, blood-free solids. Since the average total increase of the potassium was 7.3 mM per 100 gm. of fat-free, blood-free solids, the greater part of the increase in potassium in the liver must be in the cells.

TABLE VI

Effect of Increase in Total Body Water and Potassium on Extracellular Fluid (F) and Intracellular Water $\{H_2O\}_c$* Calculated from Analyses of Liver (L) of Eight Animals

	(L)			(F)			$\{H_2O\}_c$		
	Mean	σ	P	Mean	σ	P	Mean	σ	P
	gm		per cent	gm.		per cent	gm		per cent
Before injection	1000			211	±37		646	±13	
After injection	1147	±41		244	±40		694	±18	
Δ.	+147	±41	0 00	+33	±55	55	+48	±22	3 0

* $\{H_2O\}_c$ = gm. of water per kilo of intracellular phase.

DISCUSSION

The difficulty in determining whether acute edema produced experimentally in skeletal muscle following increases in total body water is less when body potassium is high than when it is normal is that potassium can be increased only moderately in body fluids because of its toxic effects. In all of the dogs the average retention of the potassium injected was 2.5 mM per kilo of body weight.

The statistical method was applied to the phase volume data of the skeletal muscle from the normal dogs (1) both before and after the injection of potassium solutions, and (2) before and after the injection of isotonic sodium chloride solutions containing no potassium. The calculated results are given in Table III. It is clear that following the increase in total body water and body potassium the increases in the extra- and intracellular phases of the muscle were not different from those found in normal dogs following the injection of balanced isotonic sodium chloride-

sodium bicarbonate solutions containing no potassium. Also, the absolute increase in the bulk of 1 kilo of original muscle was approximately the same, whether or not the body potassium was increased. The differences found are attributed to sampling. The results indicate that in normal dogs with normal kidney function there is no important quantitative difference in the relative phase volumes of skeletal muscle following an increase in total body water when $NaCl-KHCO_3$ solutions or when $NaCl-NaHCO_3$ solutions are injected. Also, the calculated percentage of water in the intracellular phase remained within the limits of error of the methods employed.

The potassium analyses of the skeletal muscle following the increase of body potassium showed no significant change when expressed in terms of concentration per unit of fat-free, blood-free solids, indicating that the concentration of potassium in the skeletal muscle remained unchanged.

Apparently, liver responds to the elevation of serum potassium and water. The analyses of fat-free, blood-free liver tissue under the present experimental conditions demonstrate that the original kilo of liver increased 147 gm. following the injections, of which 33 gm. (22 per cent) represent an increase in extracellular phase, and 114 gm. (77 per cent) an increase in the intracellular phase. At the same time, there was an increase of potassium in the liver cells; and with the retention of the potassium in the liver there was an increase in the cellular water.

The bearing of these findings upon the problem of edema has limitations. It should be noted first that all animals were normal; therefore, they had normal renal function. As a result, the excretion of the potassium was very rapid and the concentration of potassium in the serum never exceeded 8.00 mM per kilo of serum. However, with the increase of potassium in the extracellular fluids of the body and the increase in the total body water of the animal, the acute edema produced in the skeletal muscle was not different from that produced in normal animals receiving sodium chloride injections.

SUMMARY

1. Normal fat-free, blood-free skeletal muscle consists of an extracellular phase amounting to an average of 15.5 per cent, $\sigma \pm 1.9$, and an intracellular phase amounting to an average of 84.5 per cent, $\sigma \pm 1.9$.

2. Normal fat-free, blood-free liver consists of an extracellular phase amounting to an average of 21.1 per cent, $\sigma \pm 3.7$, and an intracellular phase amounting to an average of 78.9 per cent, $\sigma \pm 3.7$.

3. The percentage of water in the intracellular phase of fat-free, blood-free skeletal muscle was found to be 74.1 per cent, $\sigma \pm 0.4$; that of fat-free, blood-free liver was 64.6 per cent, $\sigma \pm 1.3$.

4. Experiments are described in which the phase volumes of skeletal muscles and liver were determined after an increase in total body water and body potassium produced by the intravenous injection of large volumes of isotonic sodium chloride solution containing potassium. The results on muscle were compared statistically with those obtained following injections of isotonic sodium chloride solutions containing no potassium. The data indicated the following conclusions.

The absolute increase in the bulk of 1 kilo of original muscle was approximately the same whether or not the injected solutions contained potassium.

After the simultaneous increase in body potassium and total body water, the additional water was distributed in the same way as the increased body water resulting from the intravenous injection of the isotonic sodium chloride-sodium bicarbonate solution. Therefore, in these experiments there was no indication of any influence of potassium upon the distribution of fluid in skeletal muscle in the normal organism.

The amount of intracellular phase of liver increased greatly, and the extracellular phase swelled appreciably. With the increase in cellular water in the liver, there was an increase of potassium in the liver cells.

There was no retention of potassium in the skeletal muscle.

BIBLIOGRAPHY

1. Fenn, W. O., Am. J. Physiol., 127, 356 (1939).
2. Hastings, A. B., and Eichelberger, L., J. Biol Chem., 117, 73 (1937).
3. Eichelberger, L., J. Biol. Chem., 128, 137 (1939).
4. Eichelberger, L., and Roma, M., J. Urol., 40, 366 (1938)
5. Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., J. Biol. Chem., 104, 149 (1934).
6. Eichelberger, L., and Bibler, W. G., J. Biol. Chem., 132, 645 (1940).
7. Darrow, D. C., Harrison, H. E., and Taffel, M., J. Biol. Chem., 130, 487 (1939).

THE INHIBITION OF CHOLINESTERASE BY MORPHINE IN VITRO

By G. S. EADIE

(*From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*)

Bernheim and Bernheim (1) have shown by the guinea pig ileum method that morphine inhibits the hydrolysis of acetylcholine by the esterase of the brain, and that under their conditions the inhibition was directly proportional to the concentration of the drug. By the titration method they found that the concentration of acetylcholine determines the amount of morphine necessary to give a specified inhibition. Kuhn and Surles (2) state that the reaction is somewhat dependent on the pH. Slaughter and Lackey (3), while they found the serum cholinesterase activity of the dog consistently lowered by morphine *in vivo*, were unable to obtain consistent effects *in vitro* on incubation of 0.5 cc. of serum with 1 mg. of morphine sulfate for 15 minutes and then determination of the cholinesterase by a continuous titration indicator method. That this was probably due to the method employed is shown by the data presented here.

EXPERIMENTAL

The method employed is essentially a continuous titration at constant temperature with a glass electrode and resembles closely that of Alles and Hawes (4), except that a Coleman pH electrometer was used instead of the Beckman meter, the temperature was 36.5°, and 0.011 N sodium hydroxide was used for titration. Usually about ten readings were taken during a 20 minute period, and the velocity calculated by the method of least squares. This was done in order to use all the readings; other methods of calculation would eliminate all but the first and the last. The rates are expressed in terms of cc. of 0.01 N alkali used (or acetic acid

597

produced) per minute. The experimental error was usually less than 5 per cent. The source of cholinesterase was dog serum.

At first considerable difficulty was experienced in obtaining consistent results; this was finally found to be due to spontaneous alterations of the esterase of serum kept *in vitro*. For example in 0.16 M acetylcholine at pH 7.4, 0.1 cc. of serum drawn from the dog the previous day and kept in the ice box produced hydrolysis at a rate of 0.051 cc. per minute; if, however, it was allowed to stand in the laboratory for 10 minutes before use, the rate increased to 0.55 cc. per minute. (A repetition of this several hours later, the serum being kept meanwhile in the ice box and removed 10 minutes before the experiment, gave 0.54 cc. per minute.) The increase in activity occurs also in the ice box, but more slowly; the same serum next day gave a rate of 0.053 cc. per minute without the preliminary exposure to room temperature. This increase is followed by a decrease; serum left in the laboratory for half an hour fell in activity from 0.053 to 0.048 cc. per minute, and a similar change occurs, but more slowly, at a lower temperature. In all the experiments reported, therefore, the serum was kept in the ice box, and samples removed and immediately added to the titration mixture which had previously been warmed to 36.5°. When this precaution was taken, consistent results were uniformly obtained. The cause of this change in activity was not investigated further.

Effect of Morphine on Activity-Enzyme Concentration Relationship—This relationship is known to be the usual linear one from the work of Glick (5) and it is also linear in the presence of morphine as shown in Fig. 1. The straight lines here are calculated from the equations

$$v = 0.030 + 0.164e$$

and

$$v' = 0.020 + 0.127e$$

where v and v' are the velocities in the absence and presence of morphine respectively, and e is the number of cc. of serum. The effect of morphine, then, is to alter the slope of the line, presumably by inhibiting the same proportion of enzyme at all enzyme concentrations. It may be noted that on extrapolation to zero enzyme concentration the rate of hydrolysis which here is
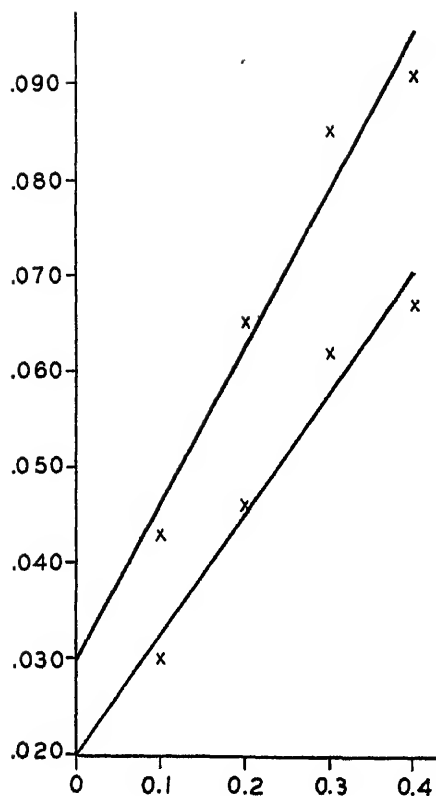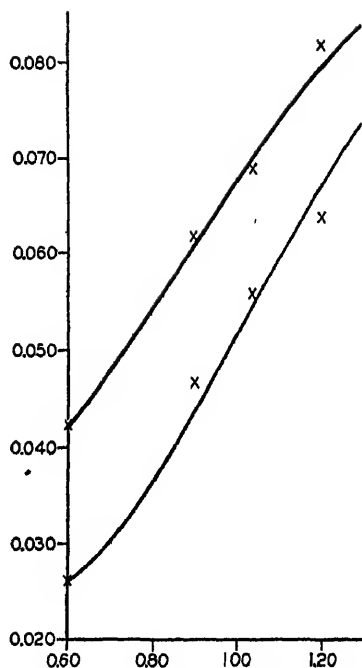
FIG. 1. The rate of hydrolysis of 0.04 M acetylcholine at pH 7.40 with varying amounts of serum in the absence (upper line) and presence of 0.0021 M morphine hydrochloride (lower line). The ordinate gives the rate in cc. of 0.01 N acetic acid produced per minute; the abscissa, cc. of serum in a 25 cc. digestion mixture. Temperature, 36 5°. The experimental points are marked.

FIG. 2. The rate of hydrolysis of varying concentrations of acetylcholine by 0.1 cc. of serum in the absence (upper line) and presence of 0.0021 M morphine hydrochloride (lower line). The ordinate gives the rate in cc of 0.01 N acetic acid produced per minute in a 25 cc. digestion mixture; the abscissa, the logarithm of the acetylcholine concentration in moles per liter multiplied by 100. Temperature, 36.5°. The experimental points are marked.

due to hydroxyl ion is apparently not the same in the presence of morphine as in its absence; this difference can be shown by statistical methods not to be significant, and is therefore to be attributed to experimental error. The difference in slope, however, is significant.

Effect of Morphine on Activity-Substrate Concentration Curve— Glick (5), using human serum at a lower temperature, found the activity-pS relationship to follow the equation of Michaelis and Menten (6), with the constant K_M 0.0011.[1] Fig. 2 shows that the same relationship holds in dog serum. The curve is calculated from the equation

$$v = \frac{V \cdot x}{K_M + x}$$

where v is the velocity as before, V the maximum velocity (here 0.114), x the substrate concentration, and K_M has the value 0.0685. This value shows a considerable variation with the aging of the serum, but since

$$K_M = x\left(\frac{k \cdot e}{v} - 1\right)$$

where e is the enzyme concentration, this variation is probably to be attributed to destruction of the enzyme.

Inhibitors may produce their effect either by uniting with the enzyme so as to destroy its catalytic activity without being displaced from it by the substrate or by competing with the substrate for the enzyme. In the former case the alteration is in V, in the latter K_M becomes $K_M (1 + f/K_f)$, where f is the molar concentration of the inhibitor, and K_f the dissociation constant of the inhibitor-enzyme compound. In the case of morphine the latter possibility is realized: 0.0685, the value of K_M in the absence of morphine, now becomes 0.167. From this, K_f may be calculated to be 0.00146. It follows from the equation that at low concentrations of substrate the inhibition from morphine is much greater.

Effect of Varying Concentrations of Morphine on Rate of Hydrolysis— Theoretically this relationship is given by the equation

$$v = \frac{V \cdot x}{x + K_M(1 + f/K_f)}$$

[1] The symbols used are those of Haldane (7).

and the curve in Fig. 3 has been plotted from this with $K_f =$ 0.00146 and $K_M = 0.0108$ (this was a fresher sample of serum than the preceding). Again the correspondence between the theoretical
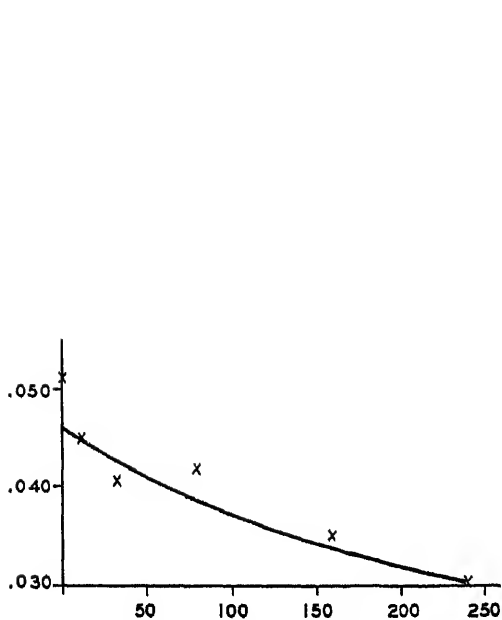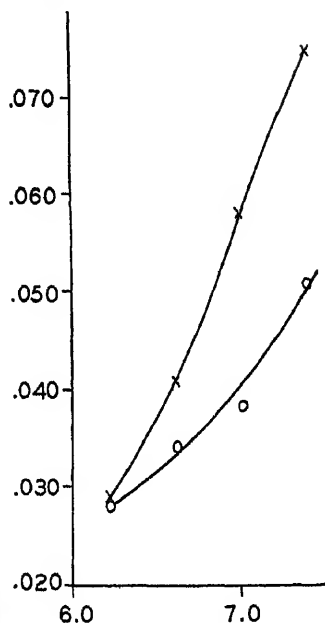
Fig. 3   Fig. 4

FIG. 3. The effect of increasing morphine concentrations on the rate of hydrolysis of 0.08 M acetylcholine by 0 1 cc. of serum. The ordinate gives the rate in cc. of 0.01 N acetic acid produced per minute in a 25 cc. digestion mixture; the abscissa, the concentration of morphine hydrochloride in mg. per 100 cc. Temperature, 36 5°. The experimental points are marked

FIG. 4. The effect of pH on the hydrolysis of 0.04 M acetylcholine by 0.4 cc. of serum in the absence (upper line) and presence of 0.0021 M morphine (lower line). The ordinate gives the rate in cc. of 0.01 N acetic acid produced per minute in a 25 cc. digestion mixture, the abscissa, the pH. Temperature, 36.5°. The experimental points are marked

and actual values is satisfactory, and this confirms the conclusions reached above.

Effect of pH—There is no satisfactory theoretical treatment of this complicated relationship. The results are shown in Fig. 4,

but the curves have no theoretical significance.   The velocities have been corrected by subtracting the values for hydrolysis by hydroxyl ion at each pH.   The percentage inhibition by morphine appears to be approximately constant over the range pH 7.4 to 7.0 but beyond this it decreases rapidly and is almost absent at pH 6.2.

SUMMARY

The inhibitory effects of morphine on the hydrolysis of acetylcholine by the esterase of dog serum under varying concentrations of enzyme, substrate, and inhibitor are discussed.   They have been shown to follow the theoretical form demanded by the hypothesis that both substrate and inhibitor combine with the enzyme and compete for it.   The effects of pH on this inhibition have also been described.

BIBLIOGRAPHY

1. Bernheim, F., and Bernheim, M. L. C., *J. Pharmacol. and Exp Therap.*, **57**, 427 (1936).
2. Kuhn, H. H., and Surles, D., *Arch. internat. pharmacod.*, **58**, 88 (1938).
3. Slaughter, D., and Lackey, R. W., *Proc. Soc. Exp. Biol. and Med.*, **45**, 8 (1940).
4. Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, **133**, 375 (1940).
5. Glick, D., *Biochem. J.*, **31**, 521 (1937).
6. Michaelis, L., and Menten, M., *Biochem. Z.*, **49**, 333 (1913).
7. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London and New York (1930).

A SPECTROCHEMICAL DETERMINATION OF SODIUM IN BLOOD SERUM*

By L. T. STEADMAN

(From the Department of Radiology, School of Medicine and Dentistry, The University of Rochester, and the Strong Memorial Hospital, Rochester, New York)

A spectrochemical method for the quantitative analysis of sodium in blood serum has been worked out and used during the past 2 years for routine analyses. It differs in several respects from the methods described by Thomson and Lee (1), Langstroth (2), and Duffendack *et al.* (3) for measuring sodium in body fluids. The first two require specially constructed sparking arrangements for exciting the sample; the third employs a high voltage A.C. carbon arc. In the present method the regular low voltage D.C. carbon arc equipment is used, with only slight modification (this is manufactured as standard equipment by the Bausch and Lomb Optical Company) and a Bausch and Lomb medium quartz spectrograph. For the sake of completeness and for future reference, the method will be described in detail, although some of the procedures concerning excitation in the low voltage D.C. arc between graphite electrodes are in common use and recently have been discussed by other workers, particularly Cholak (4), Cholak and Story (5), Owens (6), and Pierce *et al.* (7).

Procedure

An accurately measured volume, 0.5 ml. or less, of serum is diluted 40 times with distilled water. For the internal standard a cadmium chloride solution is prepared containing 1 part in 4000 of cadmium. Equal amounts, 0.25 ml., of these two solutions are placed in a small silica dish and the mixture rapidly ashed on

an electrical heater unit. While still warm, the residue is re-dissolved in about 0.25 ml. of 10 per cent HCl, transferred by pipette to the crater of a piece of spectroscopic carbon, and evaporated to dryness by holding the electrode beside the heater unit. The use of much larger amounts of HCl causes a serious loss of sample because of transference into and over the carbon with repeated filling of the crater.

The carbon electrode is a piece of National Carbon Company specially pure graphite about 2.5 cm. long, 6.3 mm. in diameter, with a crater 4 mm. in diameter and 6 mm. in depth. The regular spectroscopic electrodes of the same company are not suitable, presumably because of impurities which interfere with the intensity of the sodium line that is used for measurement. The electrode is connected to the negative side of the power line and is placed below the positive electrode, which is a 3.5 cm. piece of the regular variety of graphite. The positive electrode also has a crater which keeps the discharge from running up the outside of it. A simple attachment is provided for the Bausch and Lomb arc stand to hold the electrodes in line at an angle of about 15° with the vertical, the upper electrode being nearer the spectrograph. The negative electrode containing the sample may be used again for a second sample, the positive electrode four times. The arc is supplied by a 115 volt D.C. generator. The current is initially adjusted to read 6 to 7 amperes by means of the series resistor. A fresh electrode is burned for 20 seconds before the sample is introduced and for 75 seconds while the sample is arced and the photographic exposure made.

The light from the negative electrode and its immediate neighborhood is projected in an approximately parallel beam onto the slit of the spectrograph by means of a 7.6 cm. focal length quartz plano-convex spherical lens. The arc is 60 cm. from the slit. With appropriate separation of the electrodes, the light from the positive electrode falls below the slit. As the hot spot moves around the rim of the crater of the lower carbon, the inclination of the electrodes prevents the light emitted in the direction of the optical axis of the spectrograph from being cut off by the electrode itself. The width of the slit is 0.01 mm. In front of the slit is placed a rotating sector consisting of a half cylinder milled in a steel rod 9.5 mm. in diameter. This arrangement constitutes an

adaptation of the method for measuring relative intensities described by Hasler and Lindhurst (8) for use with a grating spectrograph. The spectrum lines that are measured in the analysis of sodium are the unresolved pair of sodium lines of 2680.3 and 2680.4 Å. and the cadmium line of 2677.6 Å. The background intensity in this region is not troublesome. By means of an adapter, a short piece of 35 mm. positive motion picture film is inserted in the plate holder, covering only the region of measurement, and two exposures are made. The photographic processing is done in the usual way.

Interpretation of Spectrum Plate

The half cylinder sector produces a spectrum line which decreases in density from both ends toward the center, so that there are two extinction points whose separation depends on the intensity of the light striking the photographic emulsion. The distance L between the two extinction points is measured by means of a Bausch and Lomb spectrum-measuring magnifier. For this type of sector the intensity of a spectrum line in the source is inversely proportional to the distance between the two extinction points for small values of the ratio $L:2R$, where $2R$ is the diameter of the sector. For the quantities of normal serum and cadmium solutions given above, the spectrum lines are of nearly the same intensity and L is about 1.5 mm.

The working or calibration curve is shown in Fig. 1. In the construction of this curve a solution of NaCl, KCl, $CaCl_2$, $MgCl_2$, and $NaH_2PO_4 \cdot H_2O$ was prepared equivalent in inorganic content to normal serum, and the ashing and arcing procedures mentioned above were carried out. The value of sodium concentration in normal human serum was taken as 335 mg. per 100 ml. of serum. Shohl (9) gives the preferred value as 330 mg. per 100 ml. of serum, but it is immaterial as far as the calibration curve is concerned which value is used. The NaCl content was then changed to give the sodium values listed along the axis of abscissas. Each point is an average of eight like determinations.

The possible uncertainty in the reading of L is about ± 0.05 mm., for $L = 1.5$ mm., so that the determination of a ratio of intensities is accurate to ± 5 per cent. The error is substantially the same for a 50 per cent increase or decrease in intensity. The

error in an intensity determination introduced because of the appreciable magnitude of $L:2R$ is less than 0.2 per cent.   Larger
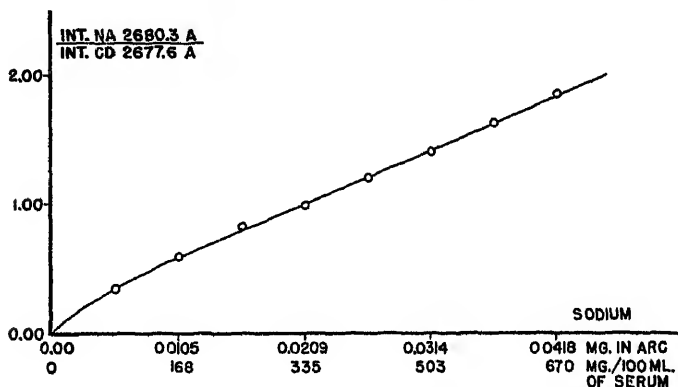
Fig. 1. Working curve for analysis of sodium.   The ratio of the intensity of the Na line in the source to the Cd line is plotted against the quantity of Na placed in the arc electrode.

TABLE I

Duplicate Analyses of Serum for Sodium by Chemical and Spectrochemical Methods

The results are expressed in mg. of sodium per 100 ml. of serum.

Spectrographic analysis	Chemical analysis	Per cent difference
312	304	−2.5
313	321	+2.5
332	322	−3.0
328	327	−0.3
302	307	+1.6
291	280	−3.7
305	311	+2.0
298	297	−0.3
321	312	−2.8
291	286	−1.7
302	304	+0.7
299	296	−1.0
Average..		−0.7

fluctuations in intensity appear, however, because of variations occurring in the process of ashing and arcing.   These fluctuations

in intensity are random in character. The standard deviation for a series of 80 determinations on like samples of serum was found to be ±8 per cent. In practice, to obtain a measurement of sodium with a standard error not greater than ±3 per cent, it is customary to make six determinations and compute the average. These may be done in 1 hour. As a comparison between this spectrochemical method and a well known chemical method, the analyses of a number of serum samples from fever patients are presented in Table I. The chemical analyses were made by Dr. H. E. Thompson, Jr., of the Department of Obstetrics and Gynecology, using the uranyl zinc acetate method of Barber and Kolthoff (10) as modified by Salit (11). The agreement is within the standard error, about ±3 per cent for both methods.

<div align="center">DISCUSSION</div>

The spectrochemical method described here is of a general nature and is adaptable to the measurement of sodium in other biological materials. The conditions for maximum sensitivity were sought in order to give a method requiring a minimum of material. The influence on the sodium-cadmium ratio of positive elements other than sodium would have to be investigated in each case. However, for blood serum the other elements are in such relatively small proportions that the curve shown is the same, in the region of normal sodium, when only sodium and cadmium are present in the test sample. Small amounts of hemolysis also do not affect the measurement. Furthermore, the method may be simplified for serum by omitting the ashing, and introducing a mixture of the serum and cadmium solutions directly into the electrode. The precision of the method is thereby somewhat increased and the time for an analysis materially reduced.

Several other experiments were carried out with test solutions to determine the validity of the method of measurement under various conditions differing considerably from those discussed above. The intensity ratio for two values, 1.00 and 1.84, was found to be the same before and after reducing by 53 per cent the intensity of the light from the arc by means of a wire screen placed over the projection lens. This result shows that the whole procedure of measuring relative intensities, involving sector,

photographic emulsion, processing, and measuring by eye is in practice reliable over a good range of intensities. The ratio of intensities is independent of a 50 per cent increase in the arc current. If the amounts of sodium and cadmium in a sample are uniformly increased or decreased, the ratio of intensities remains the same. The intensity ratio is about equally sensitive to changes in cadmium as to changes in sodium. All of these results are established of course only within the experimental error. The intensities of the sodium and cadmium lines in themselves, however, are mutually dependent in some degree on the absolute amounts of each in the arc, and the calibration curve shown does not necessarily represent the variations in sodium line intensity with quantity of sodium in the arc. Also, the calibration curve and some of the results described above concerning conditions in the arc may be somewhat different if other sodium or cadmium lines are chosen for measurement.

SUMMARY

A spectrochemical method for the quantitative analysis of sodium in blood serum is described which, for the most part, requires only commonly used spectrographic equipment and laboratory facilities. A determination with a standard error of ±3 per cent may be made in 1 hour on a sample of 0.5 ml. or less. The useful range is from 5 to 50 γ of sodium. The method is applicable to the measurement of sodium in other biological materials.

BIBLIOGRAPHY

1. Thomson, K. B., and Lee, W. C., *J. Biol. Chem.*, **118**, 711 (1937).
2. Langstroth, G. O., Proceedings of the 5th (1937) summer conference on spectroscopy and its applications, New York, 113 (1938).
3. Duffendack, O. S., Thomson, K B., Lee, W. C., and Koppius, O. G., *J. Biol. Chem.*, **126**, 1 (1938).
4. Cholak, J., *Ind. and Eng. Chem., Anal. Ed.*, **7**, 287 (1935).

5. Cholak, J., and Story, R. V., *Ind. and Eng. Chem., Anal. Ed.*, 10, 619 (1938).
6. Owens, J. S., *Ind. and Eng Chem , Anal. Ed.*, 11, 59 (1939)
7. Pierce, W. C , Torres, O. R., and Marshall, W W., *Ind. and Eng. Chem , Anal. Ed.*, 12, 41 (1940).
8 Hasler, M. F., and Lindhurst, R W., *Rev Scient. Instruments*, 7, 137 (1936)
9. Shohl, A T., Mineral metabolism, New York, 22 (1939)
10. Barber, H H , and Kolthoff, I. M., *J. Am Chem. Soc.*, 50, 1625 (1928).
11. Salit, P. W., *J. Biol. Chem.*, 96, 659 (1932)

.

THE SPECTROCHEMICAL DETERMINATION OF BIS-MUTH IN BIOLOGICAL MATERIAL*

By L. T. STEADMAN AND H. E. THOMPSON, JR.

(*From the Department of Radiology and the Department of Obstetrics and Gynecology, School of Medicine and Dentistry, The University of Rochester, and the Strong Memorial Hospital, Rochester, New York*)

The need for a quantitative method for the determination of submicrogram quantities of bismuth in body fluids of patients undergoing antiluetic treatment led the authors to develop the spectrochemical method of analysis which is described in this paper. This method is based upon the spectrochemical procedure for bismuth devised by Cholak (1) by which it was possible to measure down to 0.001 mg., with an average error of ± 20 per cent. With alterations in the chemical part of the procedure and application of the spectrographic technique of Steadman (2), as employed in the spectrochemical determination of blood serum sodium, it was found possible to measure bismuth in amounts down to 0.00001 mg. with comparable accuracy.

Preparation of Sample

The biological material is measured into a glazed porcelain crucible of appropriate size and evaporated to dryness on a steam bath, after which it is placed in a cold electrical muffle furnace and then heated overnight to not over 500°. At the end of this period of ashing any residue of carbon may be oxidized by the addition of a few tenths of a ml. of concentrated nitric acid, evaporation just to dryness, and reheating for a short time in the hot muffle. If, at the completion of ashing, any red color due to ferric oxide is apparent, 1 ml. or so of concentrated nitric acid

should be added and the whole evaporated to dryness on the steam bath in order to convert the iron to a soluble form.

The ash is dissolved in 0.1 ml. of concentrated nitric acid and water, and quantitatively transferred to a 16 × 150 mm. test-tube and the pH adjusted as recommended by Cholak. That is, the ash solution is neutralized to methyl orange with 20 per cent sodium hydroxide, and (1 + 2) hydrochloric acid added until acid to methyl orange; then 0.6 ml. more acid is added. The volume of the solution is then made to 10 ml. with distilled water.

The solution is saturated with hydrogen sulfide overnight and the precipitate filtered out, with a sintered glass filter. Jena No. 1bG3 and Ace glass filters of porosity D have been used and found satisfactory for this work. However, they should be employed with little or no suction, since otherwise the extremely small amount of precipitate would be lost.

The precipitate is rinsed with 2 × 2 ml. of 0.1 per cent hydrochloric acid saturated with hydrogen sulfide and dissolved in 4 × 1 ml. of hot (1 + 1) nitric acid. This is followed by a rinse with hot water. The filtrate is transferred to a small porcelain crucible, 1 ml. of the standard zinc solution added, and the solution evaporated to dryness and subjected to spectrographic analysis as described below. If a considerable amount of bismuth appears to be present from the size of the precipitate, the filtrate is made to an appropriate volume and aliquots are taken, and to these are added the usual amount of the standard zinc solution.

Spectrographic Procedure

The spectrographic procedure for analyzing the prepared sample for bismuth follows closely the method referred to above for sodium (2). In the sodium method the sample is placed in the crater of the negative graphite electrode of a low voltage direct current arc. When the sample is being excited, the light from the negative electrode and immediate surroundings is projected onto the slit of a Bausch and Lomb medium quartz spectrograph. A half cylinder sector, after Hasler and Lindhurst (3), placed in front of the slit permits the intensity of a selected sodium line to be compared with a nearby cadmium line used as the internal standard. Exposures are made on 35 mm. positive motion picture film and the extinction points of the spectrum lines are measured

with a low power microscope and scale. Only those details of the method which have been modified in dealing with bismuth will be described here.

The sample is redissolved in two washings of 0.1 ml. each of concentrated nitric acid, rapidly dried in the spectroscopic carbon, and arced in the same way as described. The internal standard and the spectrum lines chosen for measurement were the same as those selected by Cholak. The bismuth line of 3067.7 Å. is compared with the zinc line of 3035.8 Å. The presence of the iron line of 3067.3 Å. impairs or even destroys the accuracy of
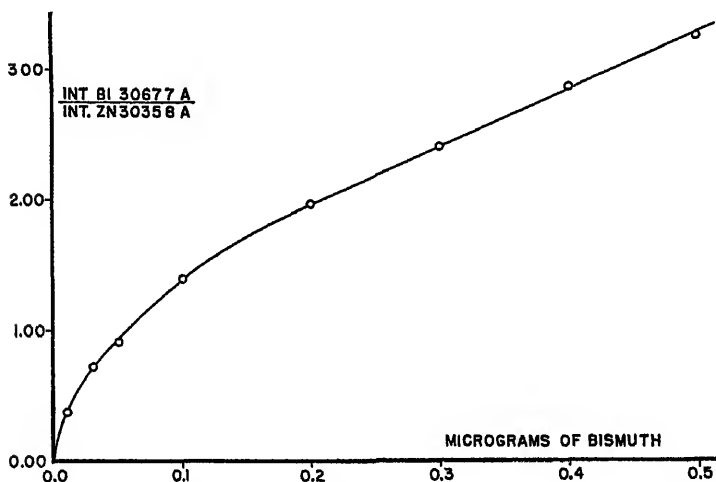
Fig. 1. Working curve for analysis of bismuth. The ordinate indicates the ratio of the intensity of the Bi line in the source to the Zn line.

measurement of intensity of the bismuth line in this method as well, except when the iron line is definitely weaker in intensity. The preparation of the sample described above, however, reduces any iron originally present to such a small amount that the iron line is of less intensity than is the bismuth line for 0.01 γ of bismuth. As in the sodium method, National Carbon Company specially pure graphite is used for the negative electrode, regular spectroscopic graphite of the same make being used for the positive electrode. The spectrum plate of the electrodes alone sometimes shows bismuth, but less than 0.005 γ.

The calibration or working curve shown in Fig. 1 was prepared by arcing varying amounts of bismuth in the presence of 0.1 mg. of zinc. Each point is an average of eight determinations. The bismuth and zinc in solution were first measured into crucibles, evaporated to dryness, and the residue, without being heated at high temperatures, was redissolved and transferred to the spectroscopic electrode. The bismuth standard solution was prepared by dissolving a weighed quantity of metallic bismuth in nitric acid. The standard zinc solution was made by dissolving a weighed amount of zinc acetate in water and acidulating with hydrochloric acid.

<div align="center">DISCUSSION</div>

The biological material is incinerated so that the use of reagents necessary for wet ashing may be eliminated. It is important that the temperature shall not exceed 500° because of a serious loss of bismuth at somewhat higher temperatures. For instance, when 0.3 γ of bismuth was added to 5 gm. of tissue and the ashing carried out at 550°, the recoveries were only 16 to 30 per cent of the expected value, while ashing at 500° yielded on the average a 94 per cent recovery.

The precipitation of bismuth sulfide is carried out in test-tubes, and the use of a manifold attached to the hydrogen sulfide supply makes it a simple matter to carry out precipitations on sixteen samples at once with a single Kipp generator.

The use of filter paper for the separation of bismuth sulfide from the supernatant liquid was abandoned in favor of sintered glass filters because of the inability to find a filter paper that was sufficiently free of bismuth. The glass filters are cleaned after each use by sucking through water in the reverse direction, rinsing with concentrated nitric acid and finally with distilled water. It is customary to follow the washing of glass and ceramic ware used in this procedure with immersion for an hour or more in nitric acid, followed by a rinse with distilled water. Blank runs which were made on the complete procedure to test the reagents and glassware consistently showed less than 0.005 γ of bismuth to be present.

The intensities of the bismuth and zinc lines are about equal for 0.057 γ of bismuth, and the distance between the two extinction

TABLE I

Bismuth Recoveries

Bismuth added	Bismuth found	Average	Bismuth expected*	Per cent recovery
γ	γ	γ	γ	
0.00	0.05			
0.00	0.06			
0.00	0.04			
0.00	0.03			
0.00	0.06			
0.00	0.06	0.05		
0.05	0.12			
0.05	0.12			
0.05	0.15			
0.05	0.06			
0.05	0.06	0.10	0.10	100.0
0.10	0.07			
0.10	0.20			
0.10	0.26			
0.10	0.09			
0.10	0.11			
0.10	0.11	0.14	0.15	93.3
0.20	0.24			
0.20	0.25			
0.20	0 20			
0.20	0.14			
0.20	0.24			
0.20	0.24			
0.20	0.27	0.23	0.25	92.0
0 30	0.26			
0.30	0.29			
0.30	0.27			
0.30	0.32			
0.30	0.31			
0.30	0.43	0.31	0.35	88.6
0.40	0.33			
0.40	0.43			
0 40	0.53			
0 40	0.45			
0.40	0.45			
0.40	0.43			
0.40	0.43	0.43	0.45	95.6
Weighted average .		. .	. . . .	93.7

* Bismuth added $+ 0.05 \gamma$ of bismuth already present in tissue sample.

points of each line is about 1.5 mm. The standard deviation computed from 60 determinations in this region of bismuth values is about ±16 per cent, but is somewhat greater when the bismuth content of the sample is 0.01 or 0.5 γ. In practice, measurements are made on samples containing bismuth in the range of 0.03 to 0.3 γ, either by taking aliquots or multiples of an initial test sample.

The intensity ratio is not unduly sensitive to changes in arc current; a 45 per cent increase in current produces about a 15 per cent increase in the bismuth to zinc ratio. If the amounts of bismuth and zinc are both increased or decreased by 50 per cent, the intensity ratio changes about 10 per cent, which is also more variable than the sodium to cadmium ratio under similar conditions. The background intensity for the bismuth line is usually higher than in the region of the sodium line of 2680.3 Å., which makes the reading of the extinction points less accurate at times. Since the ratio of intensities was found to remain essentially the same when a wire screen placed over the projection lens reduced the light intensity by 53 per cent, it was concluded that the amount of background present causes no large error and is probably taken into account in the construction of the calibration curve.

The results on a number of known bismuth samples are given in Table I. These were prepared by adding varying amounts of bismuth to 5 gm. of placental tissue and then carrying the samples through the complete procedure. It is observed that the recovery on the average is about 94 per cent. Other experiments have shown that a large part of the loss of bismuth is associated with the filtration process. The increase in the magnitude of the standard deviation in this method over the standard deviation found in the sodium method is observed to come about mainly in the preparation of the sample and not in the arcing procedure.

SUMMARY

The spectrochemical method of Cholak for quantitatively measuring very small amounts of bismuth in biological samples has been improved in sensitivity. The calibration curve covers a range of bismuth values from 0.01 to 0.5 γ. Except for the ends of the range, quantities may be measured with a standard error of ±16 per cent for a single determination.

The authors wish to express their appreciation to members of the Bausch and Lomb Optical Company, particularly Mr. M. Herbert Eisenhart, for their advice and encouragement of the work in spectrochemical analysis.

BIBLIOGRAPHY

1. Cholak, J., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 26 (1937).
2. Steadman, L. T., *J. Biol. Chem.*, **138**, 603 (1941).
3. Hasler, M. F., and Lindhurst, R. W., *Rev. Scient. Instruments*, **7**, 137 (1936).

'

AZLACTONES

III. ACYLATION OF AMINO ACIDS IN PYRIDINE*

By HERBERT E. CARTER, PHILIP HANDLER,† AND
CARL M. STEVENS

*(From the Division of Biochemistry, Noyes Laboratory of Chemistry,
University of Illinois, Urbana)*

While preparing a series of acyl derivatives of amino acids for metabolism studies, we had occasion to investigate the acylation of amino acids using pyridine as the solvent and condensing agent. Dakin and West (1) and Levene and Steiger (2) found that the action of acetyl chloride on α-amino acids in pyridine under rather drastic conditions yielded acetylamino ketones. It seemed possible that acetylated amino acids might be obtained under milder conditions. The purpose of our work, therefore, was to study this reaction at moderate temperatures in the hope of developing a method for acylating amino acids in an anhydrous medium and without using an excess of acyl halide.

It was discovered that valine, leucine, and phenylalanine react readily at room temperature with acyl chlorides in pyridine, while serine, threonine, cystine, and tyrosine are not attacked, perhaps owing to their insolubility in the solvent. The yields of acyl derivative obtained with amino acids of the first group (with equivalent amounts of the reactants) varied from 40 to 70 per cent. Increasing the amount of acid chloride had little effect on the yield. The crude products were usually oils or gummy solids and were difficult to purify. These data indicate that the

low yields of acylated amino acids were due to side reactions rather than to incomplete acylation of the amino acids. A further study revealed that the accompanying reactions (and perhaps others) may occur when α-amino acids are treated with acid chlorides in pyridine at moderate temperatures.

R—CH—CO$_2$H $\xrightarrow{\text{R}^1\text{COCl}}$ R—CH—CO$_2$H $\xrightarrow{\text{R}^1\text{COCl}}$ R—CH——C=O
| | | |
NH$_2$ NH N O
| \ /
CO C
| |
R^1 R^1

R—CH——C=O + R—CH—CO$_2$H → R—CH—C—NH—CH—CO$_2$H
| | | ‖ |
N O NH$_2$ NH O R
\ / |
C CO
| |
R^1 R^1
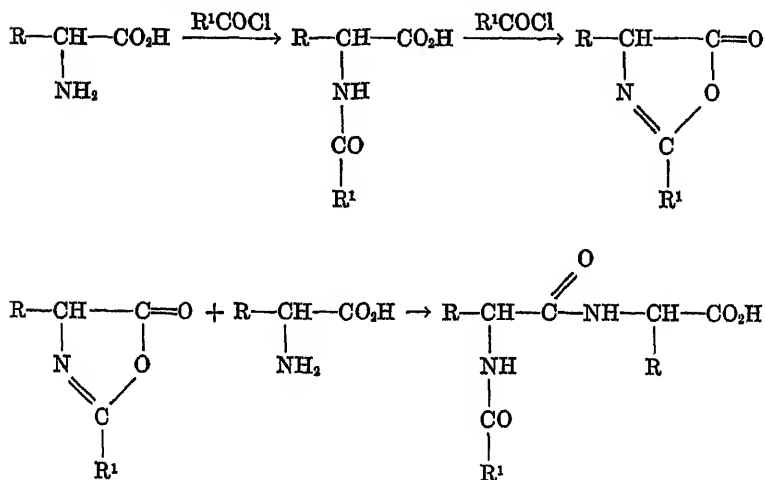
The acylated amino acid, which is first formed, may react further with the acid chloride to produce an azlactone and the latter compound may react with another molecule of amino acid to form an acylated dipeptide. The nature of the acid chloride has a marked effect on the composition of the final product. Thus benzoyl chloride is more effective than aliphatic acid chlorides in causing azlactonization. Therefore, when an amino acid is benzoylated in pyridine, very little of the simple benzoyl derivative can be isolated from the reaction product, which consists of a mixture of benzoyl derivative with benzoylated dipeptide and other products. In the case of aliphatic acid chlorides there is less tendency for the acyl derivative to react further and fair yields of the acyl derivative may be obtained. However, the difficulties involved in isolation and purification of the products render this method of acylation an unsatisfactory one, even in the case of acyl chlorides.

It seemed possible that the reaction of an azlactone with an amino acid in pyridine might furnish a good method for preparing

acylated dipeptides. However, in only one or two instances was it possible to obtain pure dipeptides in this way. The reaction products were generally gummy solids containing acylated amino acid, acylated dipeptide, and other substances.

Acylation in Pyridine—The amino acid was suspended in 10 to 20 volumes of anhydrous pyridine in a 3-necked flask equipped with a separatory funnel, a stirrer, and a short reflux condenser fitted with a calcium chloride tube. The stirrer was started and 1.1 moles of acid chloride were added dropwise from the separatory funnel. The temperature of the reaction mixture was kept below 40° by means of a water bath. In the case of *dl*-valine, *dl*-phenylalanine, and *dl*-leucine the amino acid dissolved as the acid chloride was added. The solution was allowed to stand 5 hours after the addition of the acid chloride was completed. (Pyridine hydrochloride sometimes separated during the course of the reaction.)

A small amount of water was added to the pyridine solution to hydrolyze any azlactone present and the pyridine was removed under reduced pressure. Water was added and the solution was reconcentrated under reduced pressure. (Usually the derivative separated either in crystalline form or as an oil during the concentrations.) The solution was acidified with dilute sulfuric acid and the derivative was extracted with ether. The ether solution was dried and the ether was distilled. The residue was recrystallized from benzene (butyryl, valeryl, 3-methylvaleryl derivatives), or from aqueous alcohol or ethyl acetate (acetyl and isobutyryl derivatives). The results of these experiments are summarized in Table I. The butyryl, valeryl, and higher acyl derivatives of amino acids retain benzene very tenaciously even *in vacuo*.

Benzoylation in Pyridine—11.7 gm. (0.1 mole) of *dl*-valine were suspended in 70 cc. of pyridine and benzoylated by the above procedure. After 6 hours stirring, water was added to the reaction mixture, and the pyridine was removed under reduced pressure. The residue was acidified strongly with hydrochloric acid, precipitating an oil which slowly solidified to a gummy solid. This was dried and extracted with hot petroleum ether, removing

4.0 gm. of benzoic acid. The residue was recrystallized from aqueous alcohol, giving 4.0 gm. of white solid melting at 170–205°. This product was insoluble in water, completely soluble in alkali, gave a neutral equivalent of 323, and yielded benzoic acid and valine on hydrolysis. These data indicate that the material was a mixture of the two possible racemic benzoylvalyl-valines (neutral equivalent, 320). The alcohol filtrate was concentrated to a syrup. This was crystallized from 200 cc. of benzene, yielding 4 gm. of material melting at 120–140° and giving a

TABLE I

Acylation in Pyridine

Amino acid	Acid chloride	Derivative					
		Yield	Neutral equivalent		N analyses		M.p.
			Calcu-lated	Found	Calcu-lated	Found	
		per cent			*per cent*	*per cent*	*°C.*
dl-Phenylal-anine	Acetyl	40					150–151
	Butyryl	60	235	234	5.96	5.98	86– 87
	Isobutyryl	70	235	234	5.96	5 88	105–106
	Valeryl	60	249	251	5.63	5.59	84– 85
	3-Methylvaleryl	70	263	265	5.32	5.35	129–130
	Trimethylacetyl	50	249	250	5.63	5.71	124–125
dl-Valine	Acetyl	40					142–143
	Butyryl	60	187	188	7.49	7.53	148–149
	Isobutyryl	65	187	185	7.49	7.49	165–167
	Valeryl	70	201	203	6.96	6.84	105–106
	3-Methylvaleryl	70	215	213	6.51	6.52	144–146
	Trimethylacetyl	55	201	202	6.96	7.01	98– 99

neutral equivalent of 254. Benzoyl-dl-valine melts at 132° and has a neutral equivalent of 221. Evidently this product was a mixture of benzoyl-dl-valine and benzoylated dipeptide. The benzene filtrate on concentration yielded a heavy oil from which no further crystalline material was obtained.

Leucine gave similar results on treatment with benzoyl chloride in pyridine.

Azlactonization in Pyridine

Benzoyl-dl-Phenylalanine Azlactone—5.38 gm. of benzoyl-*dl*-phenylalanine were dissolved in 40 cc. of pyridine. The solution

was cooled in an ice bath and vigorously shaken while 2.8 gm. of benzoyl chloride were slowly added. The solution was allowed to stand for 15 minutes at room temperature and was poured into a slight excess of iced hydrochloric acid. The precipitate was extracted as rapidly as possible with ether. The ether solution was dried and the ether removed under reduced pressure at room temperature. The residue was dissolved in 25 cc. of benzene and an equal volume of petroleum ether was added, precipitating 1.0 gm. of benzoyl-*dl*-phenylalanine. The filtrate was concentrated under reduced pressure and cooled in an ice bath, giving 1.3 gm. of benzoic acid. The filtrate was concentrated to a small volume and cooled in an ice bath. The precipitate was recrystallized from 30 cc. of hot petroleum ether, giving 3.5 gm. of benzoyl-*dl*-phenylalanine azlactone melting at 70–72°. This material produced no depression of the melting point when mixed with an authentic sample of the azlactone prepared by the method of Mohr and Stroschein (3).

The above experiment was repeated with equivalent amounts of acetyl chloride or acetic anhydride instead of benzoyl chloride. In each case, the yield of azlactone was considerably lower than that obtained with benzoyl chloride. However, a certain amount of the azlactone is hydrolyzed during the process of isolation. A more accurate measure of the amount of azlactone present was obtained by adding aniline to the reaction mixture, thus converting the azlactone to the insoluble benzoyl-*dl*-phenylalanyl anilide.

Benzoyl-dl-Phenylalanyl Anilide (3)—5.38 gm. of benzoyl-*dl*-phenylalanine were dissolved in 40 cc. of pyridine and 2.8 gm. of benzoyl chloride were added slowly with shaking and cooling in an ice bath. 5 cc. of aniline were added. The mixture was allowed to stand for 10 minutes at room temperature, and was then poured into a slight excess of iced hydrochloric acid. The precipitate was extracted twice with 50 cc. portions of warm alcohol. The residue consisted of 5.8 gm. (84 per cent) of pure benzoyl-*dl*-phenylalanyl anilide melting at 235–237°. This substance is almost insoluble in alcohol. Mohr and Stroschein (3) reported a melting point of 233° for the anilide.

The above experiment was repeated with equivalent amounts of acetyl chloride or acetic anhydride instead of benzoyl chloride. The yields of anilide were, respectively, 38 and 42 per cent of the theoretical amount.

A pyridine solution of recrystallized benzoyl-*dl*-phenylalanine azlactone was treated with aniline and worked up as described above. A very small amount of the anilide was isolated. However, the addition of 0.2 gm. of aniline hydrochloride, pyridine hydrochloride, or ammonium bromide to the reaction mixture increased the yield of anilide to 85 per cent of the theoretical. This marked effect of amine salts on the reactivity of azlactones has been encountered by Levene and Steiger (4). These authors were unable to prepare the azlactone of α-aminoisobutyric acid from a commercial sample of the amino acid containing small amounts of halogen but obtained instead an amorphous product. Halogen-free α-aminoisobutyric acid readily yielded the azlactone.

Benzoyl-dl-Alanyl Anilide—5.8 gm. of benzoyl-*dl*-alanine in 50 cc. of pyridine were treated with 4.2 gm. of benzoyl chloride and then with 5 cc. of aniline as described above. The crude product was recrystallized from aqueous alcohol, giving 5.5 gm. (69 per cent yield) of benzoyl-*dl*-alanyl anilide melting at 171–172.5°. Curtius (5) reported a melting point of 163–165° for this compound.

$C_{16}H_{16}O_2N_2$. Calculated, N 10.45; found, N 10.41

Acetyl-dl-Phenylalanyl Anilide—Acetyl-*dl*-phenylalanine in pyridine solution was treated with benzoyl chloride, acetyl chloride, or acetic anhydride and then with aniline as described above. The reaction product was recrystallized from ethyl acetate, giving the pure anilide melting at 211–212°.

$C_{17}H_{18}O_2N_2$. Calculated, N 9.93; found, N 10.01

The yields of anilide in these cases were low, ranging from 20 to 30 per cent. However, the pure, preformed azlactone (6) dissolved in pyridine and treated with aniline and aniline hydrochloride gave only a 36 per cent yield of anilide. Therefore, no conclusion may be drawn concerning the amount of azlactone produced by the action of the acid chlorides or acetic anhydride on acetyl-*dl*-phenylalanine in a pyridine solution. These results indicate that the reaction of an azlactone with an amino group is influenced by the nature of the substituting R^1 group on the azlactone ring.

n-Valeryl-dl-Valyl Anilide—5 gm. of *n*-valeryl-*dl*-valine in pyridine solution were treated with 3 gm. of valeryl chloride and

then with 5 gm. of aniline as described above. The crude product was recrystallized from aqueous alcohol, giving 1.5 gm. (22 per cent yield) of n-valeryl-dl-valyl anilide melting at 164–165°.

$C_{16}H_{24}O_2N_2$. Calculated, N 10.14; found, N 10.15

Dipeptide Formation in Pyridine

Benzoyl-dl-Phenylalanylglycine—10.8 gm. (0.04 mole) of benzoyl-dl-phenylalanine were dissolved in 50 cc. of anhydrous pyridine and 5.6 gm. (0.04 mole) of benzoyl chloride were added with cooling and stirring. After 30 minutes, 3.0 gm. (0.04 mole) of glycine were added and the reaction mixture was stirred 6 hours at room temperature. 25 cc. of water were added and the mixture was stirred for 30 minutes. The pyridine was removed under reduced pressure and the residue was treated with iced hydrochloric acid, giving an oil which slowly crystallized. The solid was removed by filtration, air-dried, and extracted with hot high boiling petroleum ether. The residue (11.5 gm.) melted at 155 - 180° and had a neutral equivalent of 295. (The neutral equivalent of benzoylphenylalanine is 269; that of benzoylphenylalanylglycine is 326.) This material was recrystallized from aqueous alcohol, yielding 3.5 gm. (26 per cent) of benzoyl-dl-phenylalanylglycine (3) melting at 225–237° and giving a neutral equivalent of 327. In addition, 3.0 gm. of benzoyl-dl-phenylalanine were obtained from the filtrates.

n-Valeryl-dl-Valyl-dl-Valine—10 gm. of n-valeryl-dl-valine were treated with 6 gm. of n-valeryl chloride and 5.8 gm. of dl-valine in the manner described above. The oily product was dissolved in ether. The solution was washed twice with water and dried. The ether was removed and the residue was extracted thoroughly with petroleum ether. The residue solidified after standing 2 days in a vacuum desiccator. It was recrystallized from aqueous alcohol, giving 3.0 gm. (20 per cent) of a nicely crystalline product melting at 173–178° and having a neutral equivalent of 301 (valerylvalylvaline, 300). This material is apparently a mixture of the two possible racemic forms of valerylvalylvaline. Two recrystallizations from benzene containing 5 per cent alcohol gave 0.6 gm. of material melting at 180–183°.

$C_{15}H_{28}O_4N_2$. Calculated. N 9.33, neutral equivalent 300
Found. " 9 18, " " 300

Several similar experiments and others with preformed azlactones were carried out.   In no case was a pure acylated dipeptide isolated from the reaction mixture.

SUMMARY

1. Certain amino acids react rapidly with acid chlorides in pyridine at room temperature.   The products are mixtures of the acylated amino acid and other substances.

2. Acyl derivatives of amino acids are converted to azlactones by treatment with acid chloride in pyridine at low temperatures.

3. Azlactones react with amino acids in pyridine, giving a low yield of acylated dipeptide.

BIBLIOGRAPHY

1. Dakin, H. D., and West, R., *J. Biol. Chem* , **78**, 91, 745 (1928).
2. Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **79**, 95 (1928).
3. Mohr, E., and Stroschein, F., *Ber. chem. Ges.*, **42**, 2521 (1909).
4. Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **93**, 581 (1931).
5. Curtius, T., *J. prakt. Chem.*, **70**, 137 (1904).
6. Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, **449**, 277 (1926).

BENZOYLATION OF AMINO ACIDS

By HERBERT E. CARTER AND CARL M. STEVENS

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

Amino acids may be benzoylated in aqueous solutions of sodium bicarbonate or sodium hydroxide. Fischer (1) first recommended the use of bicarbonate in order to avoid racemization and to improve yields of the benzoyl derivative. However, a survey of Fischer's data (1–3) reveals that the bicarbonate method accomplishes neither objective consistently. Recently Pacsu and Mullen (4) found that racemization occurred during the benzoylation of $d(-)$-alanine by the bicarbonate procedure. In this paper we wish to report certain of our experiences with the two methods.

It was observed that in the benzoylation of l-p-methoxyphenylalanine by Fischer's procedure (3 moles of benzoyl chloride per mole of amino acid in the presence of excess sodium bicarbonate) an oil remained after the odor of benzoyl chloride had disappeared. Hydrolysis of the oil yielded benzoyl-dl-p-methoxyphenylalanine and benzoic acid. The aqueous solution after separation of the oil gave the optically active derivative. When 2 moles of benzoyl chloride were used, a smaller amount of oil was produced. With 1 mole of benzoyl chloride the solution was practically clear at the end of the reaction and no racemization had occurred. In another experiment with 3 moles of benzoyl chloride the reaction mixture was stirred overnight. In this case the aqueous layer yielded a partially racemized benzoyl derivative.

The total yield of benzoyl derivative was 70 to 75 per cent with 1 mole of benzoyl chloride, and 75 to 85 per cent with 2 or 3 moles. Evidently the 1st mole of benzoyl chloride reacts mainly with amino acid, giving the benzoyl derivative. Further amounts of benzoyl chloride may then react with amino acid to give small

additional quantities of benzoyl derivative, with water to give benzoic acid, with sodium benzoate to give benzoic anhydride (5), and with the sodium salt of the benzoyl derivative to give a compound which racemizes readily. This substance is hydrolyzed slowly in the sodium bicarbonate solution and the extent of the hydrolysis determines the degree of racemization of the benzoyl derivative isolated from the aqueous layer. Fischer in several instances noted cloudy solutions and in one case obtained an oil at the end of the reaction. It seems probable that removal of the oil was responsible for Fischer's failure to encounter racemization more generally.[1]

The benzoylation of *dl*-alanine and *dl*-O-methylallothreonine in bicarbonate solution gives alkali-insoluble products which on hydrolysis yield benzoyl-*dl*-alanine and benzoyl-α-aminocrotonic acid respectively. Carter and Stevens (7) noted previously that the conversion of benzoyl-*dl*-O-methylallothreonine into benzoyl-α-aminocrotonic acid was effected by conditions which cause racemization of acylated amino acids.

As a result of these and other observations we have abandoned the use of the bicarbonate procedure and now benzoylate amino acids in an approximately 0.5 N sodium hydroxide solution as follows: 1 mole of amino acid is dissolved in 750 cc. of 2 N sodium hydroxide and 250 cc. of water are added. • The solution is treated with 2 moles (230 cc.) of benzoyl chloride and 2300 cc. of 2 N sodium hydroxide in the usual manner. The temperature of the reaction mixture is maintained below 30° by occasional cooling under the tap. When the reaction is complete, the solution is cooled in an ice bath and acidified with 340 cc. of concentrated hydrochloric acid. The precipitate is removed by filtration and, if necessary, the filtrate is concentrated under reduced pressure, cooled, and filtered. The combined air-dried product is extracted on the steam cone with 2 liters of hot high boiling petroleum ether

---

[1] Identification of the oil was complicated by the presence of benzoic anhydride. However, it seems probable that the racemizing compound is an azlactone (a type of compound which racemizes rapidly) or a mixed anhydride, which might be expected to racemize readily as a result of the activating effect of the anhydride group on the α-hydrogen atom(s) of an acid. Steiger (6) has suggested a mixed anhydride as a possible intermediate in the racemization of acetylated amino acids by acetic anhydride.

and then with 1 liter to remove benzoic acid. The crude benzoyl derivative is purified in the appropriate manner.

We have used this method in benzoylating *l-p*-methoxyphenylalanine, *l*-tyrosine, *d*-valine, *d*-, *l*-, and *dl*-threonine, *d*-, *l*-, and *dl*-allothreonine, *dl*-O-methylthreonine, *dl*-O-methylallothreonine, *dl*-phenylalanine, *dl*-alanine, *dl*-valine, the two *dl*-α-amino-β-benzylthio-*n*-butyric acids, and other amino acids. We have also prepared β-phenylpropionyl derivatives of several optically active and racemic amino acids by this procedure. The yields were consistently good and no racemization was observed.

A possible explanation of the difference between the two methods is suggested by the following experiment. Sodium benzoate (0.1 mole) was treated with benzoyl chloride (0.1 mole) in sodium bicarbonate and in sodium hydroxide solutions under the conditions used in benzoylation. The benzoyl chloride disappeared completely in 10 minutes in the sodium hydroxide solution and only a trace of benzoic anhydride was produced. In the bicarbonate solution, 45 minutes were required for disappearance of the benzoyl chloride and 0.8 gm. of benzoic anhydride was isolated. It seems probable that the more rapid rate of hydrolysis of benzoyl chloride in sodium hydroxide solution largely prevents its reaction with the sodium salt of the benzoyl derivative and the consequent racemization.

SUMMARY

The data reported in this paper indicate that benzoylation of optically active amino acids in sodium bicarbonate solution may be expected to cause a certain amount of racemization. The sodium hydroxide method is therefore recommended, since it is more convenient, gives consistently good yields of benzoyl and other derivatives, and has not led to racemization in any of the cases studied.

BIBLIOGRAPHY

1. Fischer, E., *Ber. chem. Ges.*, **32**, 2451 (1899).
2. Fischer, E., *Ber. chem. Ges*, **33**, 2370 (1900)
3. Fischer, E., and Mouneyrat, A, *Ber. chem Ges*, **33**, 2383 (1900)
4. Pacsu, E., and Mullen, J. W., 2nd, *J. Biol Chem*, **136**, 335 (1940).
5. Wedekind, E., *Ber. chem. Ges.*, **34**, 2070 (1901).
6. Steiger, R. E., *Helv. chim. acta*, **17**, 555 (1934).
7. Carter, H. E., and Stevens, C. M., *J. Biol Chem*, **133**, 117 (1940).

THE ACTIVITY OF CRYSTALLINE UREASE AS A FUNCTION OF OXIDATION-REDUCTION POTENTIAL*

By IRWIN W. SIZER AND ALFRED A. TYTELL

(From the Laboratory of Physiology and Biochemistry, Massachusetts Institute of Technology, Cambridge)

A number of different enzymes are activated by certain reducing agents (*e.g.*, KCN, H_2S, cysteine, and reduced glutathione) and inactivated by oxidizing agents (*e.g.*, $K_3Fe(CN)_6$, H_2O_2, and cystine). Under ideal conditions such inactivation is reversible by the addition of reducing agents (for a review of this subject *cf.* Hellerman (1937, 1939)). These results have been interpreted on the basis of the oxidation or reduction of substituent sulfhydryl groups in the enzyme molecule which are concerned with enzyme activity; it is proposed that the enzyme is active when the sulfur is in the reduced —SH form but inactive when it has the oxidized —S—S— configuration. In the case of urease the presence of sulfhydryl groups in the enzyme molecule has been demonstrated (Sumner and Poland, 1933) and it has also been shown that as the enzyme activity disappears there is a corresponding loss in sulfhydryl groups (*cf.* Hellerman (1939)). Sizer (1939, 1940, 1941) has demonstrated the fact that the activation energy of the urease-urea system may be modified by the presence of oxidizing or reducing agents, suggesting a change in the enzyme surface effected by these compounds. Apparently not all of the sulfhydryl groups are concerned with enzyme activity, however, for when the more readily detectable sulfhydryl groups have been rapidly oxidized with porphyrindin the urease activity is unchanged (Hellerman, 1939). With papain as well it is apparently the less reactive sulfhydryl groups which are concerned with enzyme activity (Hellerman, 1939). In the case of papain, which

behaves very much as does urease, Fruton and Bergmann (1940) question this sulfhydryl theory of enzyme action and advance the hypothesis that reducing agents form an active complex with the enzyme, thereby acting as a coenzyme. Greenberg and Winnick (1940), working with several papain-like plant proteases, on the other hand, found the sulfhydryl theory adequate to explain the action of reducing or oxidizing substances on enzyme activity.

No extensive studies have as yet been made of enzyme action as a function of oxidation-reduction potential, although such an investigation could be expected to yield considerable information on the problem of enzyme activation and inactivation. In this paper such experiments with crystalline urease and a variety of oxidizing and reducing agents are described. The rather empirical character of the observations should be emphasized.

EXPERIMENTAL

Crystalline urease was prepared according to the method of Sumner (1926) and was once recrystallized according to Sumner and Hand (1928). Saturated suspensions in distilled water were stored in the refrigerator and diluted to a suitable concentration before use. Also used in this study were several preparations of recrystallized urease prepared in the Organic Chemistry Department, Massachusetts Institute of Technology, by Mr. Walter Hughes, who used a modification of Sumner's method in the preparation of urease.

The urease activity was followed by measuring the liberated CO_2 manometrically with the method developed by Sizer (1939, 1940, 1941). A stock solution was prepared which contained 3 per cent urea, 5.4 per cent Na_2HPO_4, and 4.25 per cent KH_2PO_4. 2 ml. of this solution were placed in the reaction flask; to this were added 1 ml. of water, or of oxidant, or of reductant solution, and finally, 1 ml. of urease solution. The digest was adapted with shaking to the temperature of the water bath ($30° \pm 0.02°$) for 3 minutes before the stop-cocks were closed. Ten manometer readings were taken at minute intervals. The oxidation-reduction potential of the digest was then measured with the platinum electrode at room temperature. In several experiments the oxidation-reduction potential of the digest was followed during the course of the hydrolysis of urea, but, with the exception of the

$Na_2S_2O_4$ digest, it was found to be constant. The E_h of a digest containing sodium hydrosulfite was so unstable that it was necessary to measure the potentials at once rather than after 13 minutes. Four different experiments were run simultaneously with four manometers; one of the four was the control containing distilled water instead of an oxidizing or reducing substance, and the rates of the others were expressed as a percentage of the control.

The concentration of the oxidizing and reducing compounds was usually 0.005 M, although in some cases slightly higher or lower concentrations were used. Preliminary studies of urease activity as a function of salt concentration indicated that in general concentrations below 0.02 M were not toxic.

Results

When c.mm. of CO_2 liberated from urea by the urease is plotted against elapsed time in minutes, a linear relationship is obtained (cf. Sizer (1939, 1940, 1941)). The slope of the straight line drawn through the plotted points is taken as the measure of rate. The presence of oxidizing or reducing substances in the digest changed only the rate of the reaction and did not modify the linearity of the plot.

A preliminary survey indicated that certain substances were satisfactory for use with urease, while others, especially the heavy metal salts and many aromatic compounds, completely inactivated the urease. Eighteen different satisfactory oxidizing and reducing solutions were selected which covered the E_h range from -260 to $+540$ millivolts, and in each experiment the activity of urease was measured in each of the eighteen solutions as well as in the six controls. Urease activity was then expressed as a percentage of the control activity and plotted against E_h. In a single experiment the plotted points were widely scattered about a bell-shaped curve which resembles a typical pH-activity curve. In view of such dispersion of the plotted points nineteen independent experiments were performed with ten different preparations of crystalline urease. Similarly shaped curves with optimum activity at about $E_h = +150$ millivolts were obtained in all cases, although minor differences were apparent, the activity of certain urease preparations being affected by the range of oxidation-reduction potential to a much greater extent than that of others.

The stability of a single urease solution with reference to E_h was often found to change when the preparation was stored at 4° for several days. Hellerman (1939) has also reported that crystalline urease sometimes behaves in a "capricious" manner with reference to oxidizing and reducing compounds. It was believed at first that this variability in behavior was due to the methods used in preparing crystalline urease. Samples of recrystallized urease prepared in a somewhat different manner were obtained from Mr. Walter Hughes. These were studied in the usual manner, and the results obtained were similar in every respect to those obtained with our own urease samples.

The similarity of the general trends of the results obtained in the various experiments justified the averaging of all of the data of nineteen different experiments (twelve with our urease, seven with Hughes' urease). From these averages the standard deviations for E_h and per cent urease activity were calculated (cf. Table I). The average per cent activity was plotted against the average E_h for each of the eighteen different oxidizing and reducing solutions (Fig. 1). It is evident from Fig. 1 that even though the points are somewhat scattered they may be approximately fitted by a smooth bell-shaped curve; the optimum E_h for urease activity lies between +100 and +200 millivolts. It is apparent that urease activity is a continuous function of E_h and that there is no abrupt change in activity when mild reducing agents are added to urease partially inactivated by oxidants. Another striking and previously unreported feature of the E_h-activity curve is the fact that the enzyme activity declines at very low oxidation-reduction potentials in the presence of strong reducing agents. Too much emphasis should not be placed upon this, however, since points at low potentials were obtained with only Na_2S and $Na_2S_2O_4$. Preliminary studies by one of us (I. W. S.) on urease activity at $E_h = -370$ millivolts, in which hydrogen was bubbled into a urease solution containing platinized asbestos (this asbestos had no effect on urease activity), indicate that urease is appreciably inactivated at this low potential.

When the variability of the data, as measured by standard deviation (Table I), is examined, it is apparent that the urease activity at a given E_h is extremely variable (cf. Hellerman (1939)) and has its maximum variability in solutions of very low potential.

The oxidation-reduction potential is most variable in the middle of the E_h range studied, where the system is not as efficiently poised as at the extremes.

Examination of the plotted curve reveals the fact that the point for thiourea is unusually low; this is probably due to the fact that this substance has a slightly toxic action on the urease, or possibly

TABLE I

Activity of Crystalline Urease As Function of Oxidation-Reduction Potential of Digest Poised at Given E_h by Addition of Oxidizing or Reducing Agents

Concentration	Agent	E_h of digest	Standard deviation of E_h	Urease activity	Standard deviation of urease activity
		millivolts	*millivolts*	*per cent*	*per cent*
0.005 M	$K_3Fe(CN)_6$	+539	22	89	12
0 0025 M " +		+443	4	114	19
0.0025 "	$K_4Fe(CN)_6$				
0.005 M	"	+355	23	121	17
0.005 "	$Na_2S_2O_3$	+224	68	124	22
0.005 "	KCNS	+205	73	117	19
0.005 "	KCN	+194	80	128	22
0.005 "	Thiourea	+168	72	113	11
0.005 "	Thioglycolic acid	+61	24	122	13
0.005 "	Cysteine	+5	35	124	26
0.025 saturated	H_2S	+47	54	128	20
0.125 "	"	+8	29	125	15
0.25 "	"	−20	36	112	16
0.005 M	Na_2S	−24	25	126	21
0.01 M	"	−82	38	91	39
0.00125 M	$Na_2S_2O_4$	−89	19	105	14
0.0025 M	"	−160	17	108	16
0 005 M	"	−206	17	96	22
0.01 M	"	−257	22	86	35

because thiourea offers competitive inhibition by displacing the urease from the urea surface. The control point is also low, suggesting that in addition to E_h activation there is also some salt activation; *i.e.*, urease dissolved in distilled water has a lower activity than when in the presence of an oxidizing or reducing compound at the same E_h.

A similar E_h-activity curve with about the same optimum can also be obtained by using successive dilutions of a strongly reducing agent such as Na_2S and of a strongly oxidizing agent such as $KMnO_4$, and measuring the activity of urease in each dilution as illustrated in Fig. 2. This method has the disadvantage that concentration and toxicity effects not related to E_h may be superimposed on those related to oxidation-reduction potentials. It is
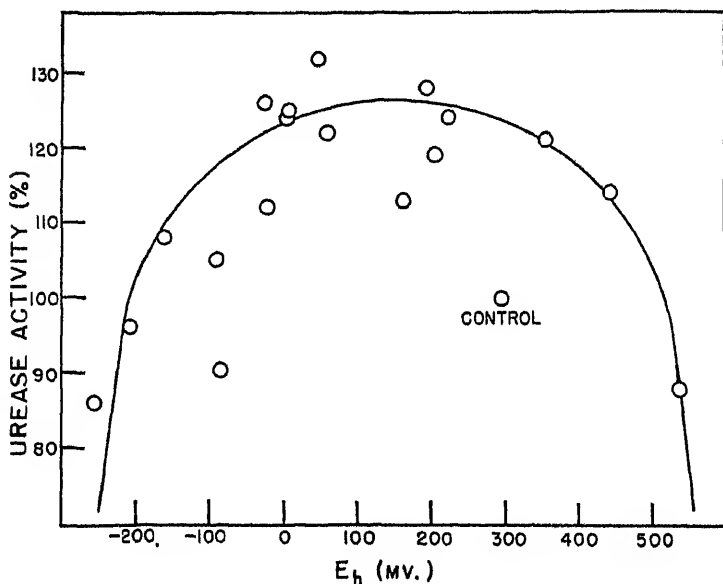
FIG. 1. Per cent urease activity is plotted as a function of the oxidation-reduction potential of the digest. The optimum E_h lies at about $+150$ millivolts. A list of the oxidizing and reducing compounds used to poise the E_h of the digest, their concentrations, and the corresponding E_h values of the digest are presented in Table I

suggested that the curve in Fig. 2 falls off more rapidly at high E_h values than does that of Fig. 1 owing to the specific toxicity of the $KMnO_4$ to urease.

A third method of investigating the E_h-activity curve is to employ mixtures of oxidants and reductants in which the cumulative concentration of oxidant plus reductant is 0.005 M. A typical example of such an experiment is presented in Fig. 3 where $K_4Fe(CN)_6$ has been mixed in various proportions with

$K_3Fe(CN)_6$ or with $Na_2S_2O_4$. The relationship between E_h and urease activity is strikingly similar (compare Figs. 1 to 3) when studied by these three different methods.
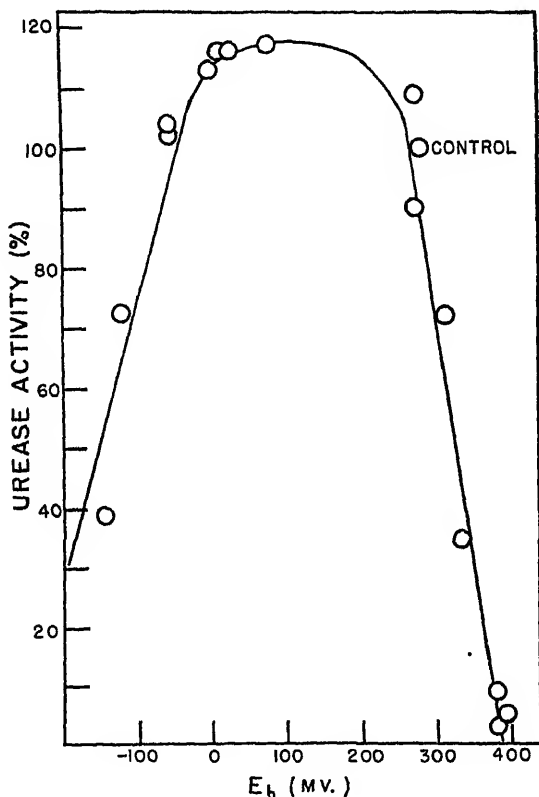
FIG. 2. Per cent urease activity is plotted against the oxidation-reduction potential of the digest   The optimum E_h is roughly +130 millivolts. The plotted points represent the urease activity and E_h of digests which contained one of the following: 0 05, 0.025, 0 01, 0.005, 0 025, 0 0012, 0.0005, 0 00025 M Na_2S (cf. points on the left side of the curve), or 0.0001, 0 00005, 0 00004, 0 000025, 0.000019, 0.000012, 0 000006 M $KMnO_4$ (cf points on the right side of the curve).

Toxicity of Oxidizing and Reducing Solutions—An examination of Fig. 1 raised the question as to whether or not the inhibitory effects of 0.005 M $K_3Fe(CN)_6$, 0.01 M Na_2S, and the several concen-

trations of $Na_2S_2O_4$ may not be related to the specific toxicity of these compounds for urease rather than to their oxidation-reduction potentials. This problem was investigated by holding the concentrations of the above salts constant and by changing the E_h of the solutions with the addition, without affecting the concentration of the original salt, of other oxidizing and reducing substances. Since the concentration of the original substance is unchanged, it can be argued that any increase in urease activity
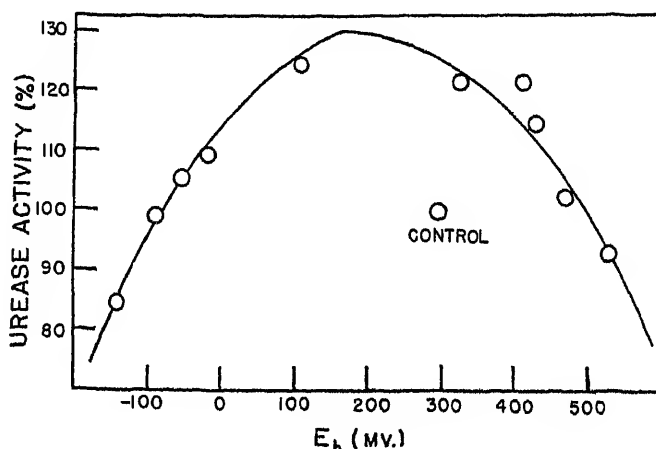
FIG. 3. Per cent urease activity is plotted against the oxidation-reduction potential of the digest which contained a total concentration of oxidant + reductant = 0.005 M. The optimum E_h is roughly +170 millivolts. Points on the negative side were obtained with digests which contained $Na_2S_2O_4$ in the following ratios to $K_4Fe(CN)_6$, 1:0, 1:4, 1:9, 3:17. The uppermost point is for a digest containing 0.005 M thioglycolic acid. For points on the positive side ratios in the digest for $K_3Fe(CN)_6$:$K_4Fe(CN)_6$ were 1:0, 3:1, 1:1, 1:3, 0:1.

effected by changing the E_h indicates that the original concentration of the oxidizing or reducing compound was not toxic. The addition of various amounts of $K_4Fe(CN)_6$, or $Na_2S_2O_4$, or Na_2S, to a urease solution containing 0.005 M $K_3Fe(CN)_6$ lowered the E_h and also raised the urease activity, although the concentration of ferricyanide was unchanged. Similarly the addition of $K_3Fe(CN)_6$ to a 0.0025 M $Na_2S_2O_4$ solution, or the addition of a solution of KCN, of $Na_2S_2O_3$, or of H_2O_2 to a 0.005 M $Na_2S_2O_4$

solution, brought about an increase in E_h and a corresponding increase in urease activity; the addition of $K_3Fe(CN)_6$ to a digest containing a 0.01 M solution of Na_2S raised the E_h and the enzyme activity. With $K_3Fe(CN)_6$ or Na_2S, studies were made of urease activity as a function of salt concentration; it appeared that these substances were toxic only at concentrations considerably greater than those employed in the E_h-activity experiments. In the case of $Na_2S_2O_4$ and Na_2S, solutions of low E_h inactivate, while those of higher E_h activate the urease; these results are not as easily explained upon the basis of the toxicity theory. From the foregoing series of experiments it appears that the effects of $K_3Fe(CN)_6$, Na_2S, and $Na_2S_2O_4$ on urease activity may be correlated, at least in part, with the oxidation-reduction potentials of these substances, rather than solely with any specific toxic effects of these salts on urease.

pH Changes—The addition of oxidizing or reducing compounds to the urease-urea digest buffered with phosphate did not appreciably modify the pH. In all cases the pH was 6.8 ± 0.1 as measured with the glass electrode.

Other Oxidizing and Reducing Compounds Used to Stabilize the Potential—Many different oxidizing and reducing compounds were tried, some of which were satisfactory, while others were not. Among the useful compounds found were cystine, methionine, glutathione, $NaClO_3$, and KNO_3, and among the unsatisfactory compounds found were heavy metal salts, many aromatic compounds, ascorbic acid, riboflavin, iodoacetic acid, and most of the dyes which were used. The results with phenolic compounds, ascorbic acid, iodoacetic acid, and dyes are essentially in agreement with those reported for urease by Quastel (1932, 1933) and by Hellerman (1939).

Experiments with Crude Urease—A 0.15 per cent suspension of Squibb urease was prepared and filtered and the effect on activity of each of the eighteen different oxidizing or reducing solutions was measured. A similar experiment was performed on a 0.4 per cent suspension of jack bean meal after filtration. With both these crude urease preparations there was no detectable action of the different oxidizing or reducing solutions upon enzyme activity. These results are in agreement with those of Fischgold (1934) who could find no relationship between oxidation-reduction po-

tential and the activity of crude urease.   Similar results on crude soy bean and *Proteus vulgaris* urease were reported by Sizer (1940, 1941).

Reversibility of Oxidation-Reduction Effects—The reversibility of the effects of oxidizing and reducing agents on urease activity has been investigated extensively by others (*cf.* Hellerman (1937)) and so this subject was not investigated in detail.   Contact of urease with strong oxidizing or reducing agents for even short periods of time resulted in irreversible inactivation.   In the usual E_h-activity experiments contact with the oxidizing or reducing solutions was so brief that irreversible inactivation did not usually occur.   Aeration of a urease solution for a few hours brings about partial inactivation which can be reversed by the addition of reducing agents (*cf.* Ganapathy (1938)).

DISCUSSION

It appears from this study that an E_h range of $+100$ to $+200$ millivolts is optimal for urease activity, and that above and below this range the urease activity decreases.   The similarity of this E_h-activity curve to the typical pH-activity curve is striking and suggests that E_h as well as pH control may be important in many enzyme studies.   It seems very likely that a similar E_h-activity relationship characterizes certain other enzymes which are activated by reductants and inhibited by oxidants.

The data on urease activity as a function of E_h are in general consistent with the sulfhydryl theory.   Hellerman (1939) has pointed out that in the urease molecule the sulfhydryl groupings differ greatly in reactivity.   It seems likely that as the E_h is decreased from $+540$ to $+150$ millivolts more and more —S—S— groupings are reduced to —SH groupings with a corresponding increase in urease activity.   At potentials below $+150$ millivolts it is possible that reduction of certain stable groupings in the enzyme (sulfhydryl, or other reducing groups essential for enzyme activity) brings about progressive inactivation of the molecule.

The data are not considered to be consistent with Fruton and Bergmann's suggestion (1940) that reducing agents activate by acting like coenzymes to form complexes with the enzyme.   The difference in activation produced by different reductants appears to be related to differences in oxidation-reduction potential rather

than to specific differences in the activities of different reductant-enzyme complexes.

The authors are very grateful to Mrs. Robert Hull, Mr. Walter Hughes, and Mr. Herbert Jaffe for assistance rendered.

SUMMARY

The relationship of the activity of crystalline urease to oxidation-reduction potential has been studied over the range from $E_h = +540$ to -260 millivolts with a large number of different oxidizing and reducing agents to poise the potential at various values. Urease activity was found to be a continuous function of E_h; a bell-shaped curve was obtained when urease activity was plotted against E_h with a maximum activity at about $E_h = +150$ millivolts. These results are interpreted in terms of the oxidation or reduction of constituent essential sulfhydryl linkages in the urease molecule by oxidants or weak reductants. The inactivation by strong reductants may be related to more extensive reduction of sulfhydryl or other groups essential for urease activity.

The activity of crude urease, unlike that of crystalline urease, is unaffected by the oxidizing and reducing agents which were used. Impurities present in the crude enzyme apparently protect the urease from the action of such agents.

Addendum—Since the completion of this work, studies have been made by one of us (I. W. S.) on the oxidation-reduction potential of the jack bean. The beans were soaked overnight in distilled water, the seed coats removed, and the potentials measured after the platinum electrode was inserted directly into the cotyledon of the bean   The average potential obtained was $E_h = +190$ millivolts. The similarity of this value with the optimum E_h for urease activity is striking, and may have some significance with reference to the normal physiology of the jack bean

BIBLIOGRAPHY

Fischgold, H., *Biochem J.*, **28**, 406 (1934).
Fruton, J. S., and Bergmann, M , *J Biol Chem.*, **133**, 153 (1940).
Ganapathy, C. V., *Current Sc* , **6**, 451 (1938).
Greenberg, D. M , and Winnick, T., *J. Biol Chem* , **135**, 761 (1940).
Hellerman, L., *Physiol Rev.*, **17**, 454 (1937); Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 165 (1939)

Quastel, J. H., *Biochem. J.*, **26**, 1685 (1932); **27**, 1116 (1933).

Sizer, I. W., *J. Gen. Physiol.*, **22**, 719 (1939), *J. Biol. Chem.*, **132**, 209 (1940); *J. Bact*, in press (1941).

Sumner, J. B, *J. Biol. Chem.*, **69**, 435 (1926).

Sumner, J. B., and Hand, D. B., *J. Biol. Chem.*, **76**, 149 (1928).

Sumner, J. B., and Poland, L. O, *Proc. Soc. Exp Biol. and Med.*, **30**, 553 (1933).

IMPROVED APPARATUS FOR THE EXTRACTION OF LIPIDS FROM LIQUIDS AND SOLIDS, WITH FURTHER APPLICATIONS TO THE FRACTIONATION OF FECAL FAT

By IRVING ALLAN KAYE, I. WALLACE LEIBNER, AND
ALBERT E. SOBEL

(From the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn,
New York)

In attempting to follow up and extend previous investigations involving the determination of lipids in biological substances with a drying and extraction apparatus (1) we encountered certain difficulties. The present modification is introduced in the hope of saving other investigators, using this apparatus and method, from similar difficulties, and to point out further uses and several added advantages of a modified apparatus with added accessories.

It was early observed that the moisture trap in the original apparatus could not be relied upon to give an accurate estimate of the amount of water contained in the sample. The water in this part of the apparatus kept increasing during the period of drying and subsequent extraction. The final weight of water added to the weight of lipids and dry residue in the thimble exceeded the weight of the original sample. Obviously water vapor must have entered the apparatus during the period of active use. Attaching a drying tube to the open end of the condenser did not remedy this condition. It was concluded that water vapor, either from the air or the water baths used to heat the separate parts of the apparatus, seeped through the ground glass joints. The same phenomenon was observed in other drying and extraction pieces of apparatus of the same type sold by different commercial concerns. The original solution to this problem was to run a blank experiment in the apparatus for the same length of time as re-

quired in a determination and subtract the amount of water found in the blank run. The dry weight, *i.e.* the combined weight of the lipid residue and material in the thimble, was found to be a more accurate index of the solid content and, indirectly, of the amount of water in the moist sample.

The same method as that used in the determination of the neutral fat fraction of feces was applied to a study of brain lipids. In this problem anaerobic extraction is to be preferred, as phospholipids, in which the brain is rich, are easily oxidized in the presence of air. To accomplish this, a T-tube was attached to the open end of the Hopkins condenser. One end was attached to a water aspirator and the other to a balloon of nitrogen. The T-tube was held in place by rubber connections; Hoffman screw clamps were inserted at these points. The apparatus was first evacuated thoroughly, then sealed off from the suction with a Hoffman screw clamp. The other clamp was then opened, permitting nitrogen from the balloon to flood the system. The procedure from this stage on was the same as that in the original method. The chief difficulty again lay in the ground glass joints, for often in a few hours the nitrogen leaked out of the system.

Both the limitations are corrected in a modified apparatus, shown in Fig. 1. A different type of joint is used; namely, a ground glass joint dipping into a pool of mercury. In addition, a 2-way stop-cock is sealed to the open end of the Hopkins reflux condenser. When an ordinary balloon containing nitrogen is attached to one part of the 2-way stop-cock, in the procedure just described, namely evacuating the system with a water aspirator after the thimble is inserted into the extraction chamber *B*, placing 100 ml. of the solvent in flask *A*, and then opening the 2-way stop-cock to the balloon, a startling contrast to the difficulties mentioned was afforded. No water blank at all was encountered and the balloon did not visibly collapse even after 3 days of extraction. The possibility of performing continuous extractions in this apparatus at reduced pressures may be mentioned, especially in connection with the extraction of heat-labile substances with a high boiling solvent.

An added advantage of these new features is the ease in freeing the apparatus of the last traces of solvent after extraction is complete and most of the solvent has been distilled over and collected

through the stop-cock of the moisture trap C. The apparatus is then completely evacuated and warm water baths at about 60° are placed around flask A and extraction chamber B. After a few hours of this treatment the residue in the thimble and the contents of the flask are solvent-free.
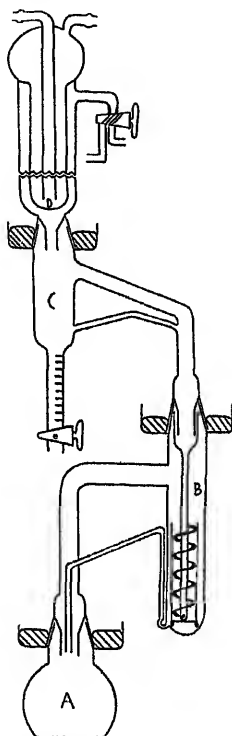
FIG. 1. Improved apparatus for the extraction of lipids

By adding a specially constructed glass thimble and spiral tube, both shown in the extraction chamber of Fig. 1, the apparatus can be used as a liquid extractor. This was originally designed to be used in the fractionation of fecal fat and seemed to operate quite successfully in a trial run wherein the neutral fat fraction of feces, containing neutral fat, unsaponifiable fat, and fatty acids, was treated with dilute aqueous sodium hydroxide to convert the fatty acids into soaps. Extraction of the aqueous layer in this

glass thimble by means of the spiral tube with petroleum ether as solvent seemed to remove the neutral fat and unsaponifiable fractions in a short time, leaving the fatty acids behind in the aqueous layer. By means of such a scheme, it is hoped a quantitative separation of the fat components of feces may be effected.

It was also found that alundum thimbles of coarse porosity could be successfully used in lipid extractions in place of the paper thimbles previously used. They are of advantage from the point of view of economy, since they can be used repeatedly. They are easily cleaned after an analysis by simply scraping out most of the residue mechanically, igniting at 500° in a muffle furnace, and washing with dilute acid followed by water. They are then dried at 110° and are ready for use when cool. Their weight remains fairly constant even after several determinations.

It has been found advisable to insulate with asbestos the side arms of the extraction chamber B and the moisture trap C. In this way less heat is required in the process of drying flask A and higher boiling liquids may be used.

Isopropyl ether, as has been pointed out previously (1) is an excellent fat solvent and can be used for drying moist substances but forms peroxides easily. If the pure solvent is used, unsaturated fats can be easily oxidized and, in addition, a heavy deposit of a brown, sweet smelling, non-lipoidal material is formed in the extraction flask. This material is always associated with the appearance of peroxides in the ether and does not appear if the solvent is peroxide-free. Recovered isopropyl ether is rendered peroxide-free by shaking with dilute sodium bisulfite solution until the ether no longer liberates iodine from acid potassium iodide solution. This usually requires only one treatment with the bisulfite solution. The solvent is then dried for a few minutes over anhydrous sodium sulfate and then distilled. The fraction boiling at 66–69° is collected. Exactly 20 mg. of hydroquinone are then added to a liter of the isopropyl ether. In the presence of this antioxidant no peroxide formation occurs on standing at room temperature or extraction at its boiling temperature. The weight of hydroquinone used does not alter to any significant extent the dry weight of the sample.

Determination of Soaps in Feces—Soaps have been determined by a modification of the method of Sabatucci Trajna (2) which

is a method quite similar to that used by Tidwell and Holt (3). In the former method, the soaps in the feces are converted to fatty acids by treatment with concentrated aqueous hydrochloric acid after the neutral fat, unsaponifiable fat, and fatty acids have been extracted. The fatty acids so formed are then extracted with benzene.

Similarly the dry residue in the alundum thimble is treated with 2 ml. of concentrated hydrochloric acid and placed immediately in the extraction chamber B containing about 15 ml. of benzene. The apparatus is then set up and about 100 ml. of benzene are added to flask A. The benzene in flask A and extraction chamber B is heated to boiling by means of external baths and the aqueous hydrochloric acid is collected in the moisture trap. About 2 hours are necessary for complete removal of the hydrochloric acid. The bath surrounding the extraction chamber is then removed and the fatty acids are extracted for about 6 hours. The benzene is removed and the apparatus freed of the solvent by the aforementioned procedure involving the use of a vacuum. The residue is then redissolved in petroleum ether and filtered according to the technique described in an earlier publication (1).

Description of Apparatus—The dimensions of the original apparatus are retained in the main in the modified apparatus. The modifications alone will be described.

The ground glass joints used in the apparatus are interchangeable 29/42 joints. The outside diameter of the mercury seal is 54 mm. A round, flat bottomed extraction flask A has been substituted for the original Erlenmeyer flask. The capacity of the flask is 200 ml. and its outside diameter at its widest point is 76 mm. The neck of the flask, extending 10 mm. from the body of the flask to the bottom of the mercury seal male joint, has an outside diameter of 28 mm.

In the extraction flask, B, the female counterpart of the joint is sealed to the outer side arm at a point 67 mm. from the lower end. The inner siphon tube extends 15 mm. below the bottom of the chamber itself. A male mercury seal joint is sealed to the top of the extraction chamber. The total length of the chamber from the top of the seal to the bottom of the chamber is 205 mm. The outside diameter of the chamber is 35 mm. The siphon tube

is bent at a 60° angle away from the chamber. The moisture trap, C, has a female mercury seal joint at the bottom of the side arm and a female joint above the graduated scale. The capacity of the graduated tube has been enlarged to 10 ml. The outside diameter of the tube is $10\frac{1}{4}$ mm. A No. 12 ground glass stopper having a 1 mm. bore is sealed to the bottom of the tube which measures 10 mm. in height from the stopper to the lower side arm. The glass tube below the stopper extends for 30 mm. and its outside diameter measures 9 mm. The length of the side arm has been shortened to 140 mm. from the center of the right angle bend to the bottom of the joint. The angle that this side arm makes with the main tube is 75°. The outside diameter of the tube above the graduated scale is 28 mm.; the tube is 60 mm. in length from the end of the narrower tube to the bottom of the joint.

The Hopkins reflux condenser, D, has a female joint sealed to it. The 2-way stop-cock attached to the condenser is a No. 12 stop-cock of 1 mm. bore. The tubing connected to it is of 9 mm. outside diameter.

The spiral tube is 210 mm. in length. The upper cup has a diameter of 19 mm. and extends for 33 mm. The outer edge of the spiral is lower than the inside edge, as the flat part must have a continuous downward slope from the center tube to the outside edge.

The glass thimble is 9 mm. wide and 90 mm. long. Three glass legs are sealed to the bottom. The alundum thimble, of coarse porosity, has the same dimensions. Both thimbles have two holes in them near the top, so that platinum wire may be drawn through them, making handling easier. The balloon, not pictured in the diagram, should be of a good quality rubber.

SUMMARY

1. A modification of the drying and extraction apparatus is described which contains an improved type of joint and a 2-way stop-cock attached to the open end of the Hopkins reflux condenser.

2. This new apparatus permits of more accurate determinations of water content.

3. Extractions can now be performed in inert atmospheres and at reduced pressures.

4. By means of specially constructed accessories, the apparatus can be used as a liquid extractor.

5. Soaps may be determined conveniently in this apparatus by a modification of Sabatucci Trajna's procedure.

6. The dangers inherent in the use of pure isopropyl ether for fat extractions are pointed out and the preparation of a peroxide-free solvent, involving the use of an antioxidant, is recommended.

BIBLIOGRAPHY

1. Kaye, I. A , Leibner, I. W., and Connor, E. B., *J. Biol Chem.*, **132**, 195 (1940).
2 Sabatucci Trajna, N., *Problema aliment.*, **3**, 118 (1939).
3. Tidwell, H. C , and Holt, L E., Jr., *J Biol. Chem*, **112**, 605 (1935-36).

STEROIDS

III. THE ISOLATION FROM EQUINE PREGNANCY URINE OF Δ-5,7,9-ESTRATRIENOL-3-ONE-17[*]

BY R. D. H. HEARD AND M. M. HOFFMAN

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada)

Recently the excretion during pregnancy in the mare of a new androstanol-3(β)-one and of allopregnanol-3(β)-one-20 was reported (Heard and McKay (2)). Further examination of equine pregnancy urine has now resulted in the isolation of another undescribed hydroxy ketonic steroid, which has been proved to be Δ-5,7,9-estratrienol-3-one-17 (formula (I)), a bond isomer of estrone in which Ring B instead of Ring A is benzenoid.

The neutral hydroxy ketones were separated from urine in the manner previously described (2), and fractionally distilled *in vacuo*. The oil collected from 170–210° was divided into a digitonin-precipitable and a digitonin-non-precipitable fraction. Treatment of the latter with semicarbazide gave a mixture of gelatinous semicarbazones, decomposing at 220–224°, which resisted purification by the usual methods. Hydrolysis of the semicarbazones led to an orange oil, which could not be induced to crystallize, but which, on acetylation, yielded well defined plates melting sharply at 158° after two sublimations and two recrystallizations. Benzoylation proved a more satisfactory means of isolation because of the sparing solubility of the benzoate (m.p. 196–198°) in the ordinary organic solvents. Saponification of the acetate and of the benzoate gave the same product, which, after purification by sublimation and repeated recrystallization, had a melting point of 138–139.5°, $[\alpha]_D$ +59°. The compound crystallized in a mixture of needles and characteristic monoclinic prisms

---

[*] A preliminary announcement has previously been made (1)

which could be separated mechanically and which melted at the same temperature. When an aqueous ethanolic solution was seeded with the latter modification, the whole crystallized in that form (Fig. 1). In all about 160 mg. of pure substance were obtained from approximately 10,000 gallons of urine. This does not, however, represent the amount excreted, owing to loss in purification and the unavoidable scattering of material in exploratory work among many fractions, all of which have not yet been examined.

Analysis of the compound and several of its derivatives clearly established the empirical formula, $C_{18}H_{22}O_2$. That 1 oxygen
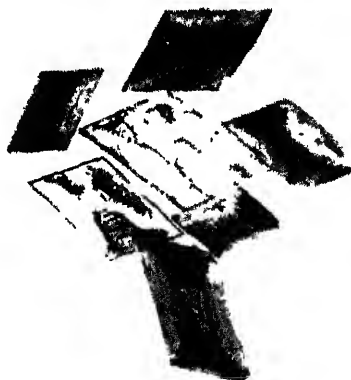
Fig 1. Monochnic crystals of Δ-5,7,9-estratrienol-3-one-17 (from aqueous ethanol).

atom was contained in a non-phenolic hydroxyl group was apparent from the insolubility of the substance in aqueous sodium hydroxide solution and the fact that it gave a monoacetate; the ketonic character of the 2nd oxygen atom was proved by the formation of a monooxime (m.p 195 -197° with decomposition). With tetranitromethane, an intense yellow coloration was produced immediately, and with nitric acid in the cold, an orange-yellow color rapidly developed. Since the absence of isolated ethylenic linkages was demonstrated by bromine titration (observed 0.06 ethenoid linkage), the unsaturation to tetranitromethane and the positive xanthoproteic reaction were obviously

due to the presence of a benzenoid ring. This was confirmed by the ultraviolet absorption spectrum (Curve A, Fig. 2), which further indicated that the aromatic ring occupied a central rather than an end position in the nucleus. The main absorption band was observed at 269.5 mμ, with a shoulder or small maximum at 278 mμ. The curve thus differs markedly from that of estrone (Curve B, Fig. 2) and unsymmetrical octahydrophenanthrene (3), but closely parallels those pertaining to symmetrical octahydro-
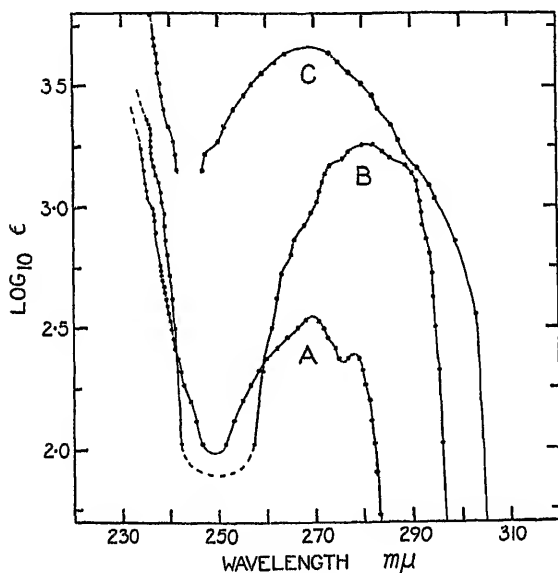
FIG. 2. Absorption spectra (in absolute ethanol) of Δ-5,7,9-estratrienol-3-one-17 (Curve A), estrone (Curve B), and Δ-3,5,7,9-estratetraenone-17 (Curve C).

phenanthrene (3) and the Ring B aromatic steroids (neoergostatriene (4), neoergosterol (5) and its epimer (6), and the Δ-5,7,9-estratrienediols-3,17 (7)). Proof of the Δ-5,7,9-estratriene skeleton and of the location of the substituents on C_3 and C_{17} was provided by the identification of the product of hydrogenation. In neutral alcohol and with the Adams platinum catalyst, 1 mole of hydrogen was taken up to yield a diol, $C_{18}H_{24}O_2$, with physical properties (m.p. 168°, $[\alpha]_D$ −5°, m.p. of diacetate 115°) in good

agreement with those of Δ-5,7,9-estratrienediol-3(β),17(α) (II). The latter was obtained by Ruzicka, Müller, and Mörgeli (8) and by David (7) on reduction of Ring A and the carbonyl group of equilenin (III) (also from 17(α)-dihydroequilenin); the Swiss investigators record a melting point of 166.5°, m.p. of diacetate 115°, and David 166–168°, $[\alpha]_D$ −16°, m.p. of diacetate 117–118°.
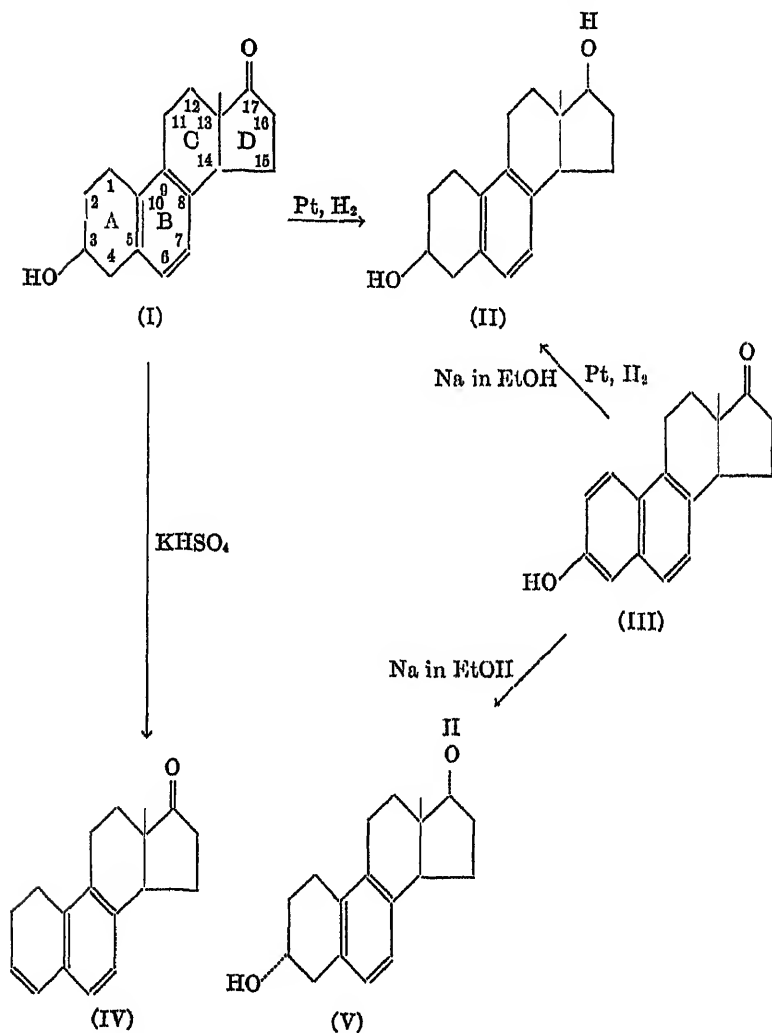
An authentic specimen of the diol (II), prepared from equilenin (III) by hydrogenation in acid ethanol (8), did not depress the melting point of that from the urinary hydroxy ketone; likewise there was no depression of melting point on admixture of the diacetates from the two sources. Since the diol (II) could result from the hydrogenation of the carbonyl group of either 3-hydroxy-17-keto-Δ-5,7,9-estratriene (I) or the corresponding 3-keto-17(α)-hydroxy derivative, it was necessary to distinguish between these two possible structures. The former was strongly indicated by the rate of color development with the Zimmermann (9) ketone reagents, which simulated more closely a 17- than a 3-keto compound, and by the magnitude of the decrease of dextrorotation on reduction of the carbonyl group to the alcohol (observed $\Delta[M]_D$ $-173°$), which is consistent with the change from a 17-keto to a 17(α)-hydroxy steroid, but greater than the values pertaining to the conversion of 3-keto to 3(α or β)-hydroxy steroids (*vide* Callow and Young (10)). Structure (I) was conclusively established when it was found that dehydration proceeded readily on heating to 150° with potassium hydrogen sulfate, conditions which effect removal of the hydroxyl group of neoergosterol (11) and 22-dihydroneoergosterol (4) with the introduction in Ring A of an ethylenic linkage in conjugation to the bonds of the aromatic Ring B. That the dehydration product (m.p. 114–116°) contained such a system was revealed by the ultraviolet absorption spectrum (Curve C, Fig. 2), which exhibited a broad maximum at 268 mμ, with $\epsilon = 4600$ (*cf*. neoergostapentaene (4, 6) and neoergostatetraene (4)). Clearly this conjugation could arise only from the 3-hydroxy compound (I), and the product must be Δ-3,5,7,9-estratetraenone-17 (IV).[1]

It is highly probable that the C_3 hydroxyl group possesses the β configuration.[2] This is evident from the work of Ruzicka,

---

[1] The ketone (IV) is undoubtedly identical with the ketone (m.p. 114–115°) isolated (as semicarbazone) by Chakravorty and Wallis (12) from the products of warm chromic acid oxidation of epineoergosteryl acetate, which contained but 1 oxygen atom and exhibited a flat absorption maximum at about 267 mμ, with $E\,_{1\,cm.}^{1\%} = 208$ (whence $\epsilon = 5300$). The authors state that, during the treatment, "the hydroxyl group at C_3 was lost and a double bond, presumably between C_3—C_4, was introduced."

[2] The prefixes α and β are here used in the sense advocated by Fieser (13) to designate the orientation of the C_3 hydroxyl group relative to the

Müller, and Mörgeli (8) and of David (7) who obtained from equilenin (III) and its 17(α)-dihydro derivative both members (II and V) of the C₃ epimeric pair of 3,17(α)-diols, which had (7) melting points of 166–168°, [α]ᴅ −16°, and 191–193°, [α]ᴅ +68°. Hydrogenation in acid ethanol yielded the lower melting isomer, while reduction with sodium in ethyl or amyl alcohol gave both diols, chiefly that with the higher melting point. Respectively the β (II) and α (V) configurations were assigned.[3] This is consistent with the generalizations that the β epimer possesses the lower melting point and more negative rotation, which relationships have been well established for steroids containing the angular methyl group on C₁₀ (Reindel and Niederländer (15), Callow and Young (10)), and which are true of the only characterized C₃ epimeric pair of Ring B aromatic steroids, neoergosterol (m.p. 152°, [α]ᴅ −11°) and epineoergosterol (m.p. 177°, [α]ᴅ +27°). Since reduction of the 17 carbonyl group of the natural compound gives rise to the diol with a melting point of 168°, it follows that the C₃—OH is β-oriented.

The characterization of Δ-5,7,9-estratrienol-3(β)-one-17 is quite irreconcilable with the conclusions arrived at by Remesov (16) concerning the structure of a highly potent estrogenic substance ("folliculosterone") derived from neoergosterol by, to quote Wintersteiner and Smith (17), "a not very clearly described procedure of oxidative degradation (lacking in characterization of the intermediary products)." Remesov formulated the compound (m.p. 252°) as (I), mainly on the basis of the non-phenolic nature of the hydroxyl group and the ultraviolet absorption spectrum. While folliculosterone could conceivably be the C₃ epimer

---

remainder of the molecule as a whole. β thus indicates the "normal" configuration as found in norsterol, neoergosterol, cholesterol, etc., and α, the "epi" configuration.

[3] From the products of reduction of equilenin (III) and 17(α)-dihydroequilenin with sodium in amyl alcohol, Marker et al. (14) isolated a Δ-5,7,9-estratrienediol-3,17(α), m.p. 172°, for which they suggested the 3(α) configuration (V) on the grounds that Windaus and Deppe (6) obtained epineoergosterol from tetradehydroneoergosterol by the same treatment. In view of the later work of David (7) who showed that both C₃ epimers result from reduction of equilenin with sodium in ethyl or amyl alcohol, and in consideration of the melting point, it would seem more probable that the diol of Marker et al. possesses the 3(β) configuration (II).

of the urinary estratrienolone, two important facts weigh against this likelihood. As Ruzicka, Müller, and Mörgeli (8) point out, the high estrogenic activity of Remesov's compound in the Allen-Doisy test (8 to 10 million I.U. per gm.) is inconsistent with the formulation. The 17(α)-diol corresponding to folliculosterone (*i.e.*, (II)) should be more potent than the ketone itself (*cf.* α-estradiol and estrone), whereas they found a much lower order of activity for both C_3 epimers (II and V) of Δ-5,7,9-estratrienediol-3,17(α) (about 250 and 120 to 150 γ per rat unit, which values correspond approximately to 40,000 and 66,000 to 83,000 I.U. per gm.). Also the absorption spectrum curve recorded by Remesov is not in agreement with the structure assigned. The light properties of Ring B aromatic steroids are well established (3–7), the main maximum occurring at 269 to 270 mμ, with ϵ_{max}. in the neighborhood of 400. Folliculosterone exhibits maximum absorption at 282 mμ, with an extinction coefficient some 10 times greater than the above value.

While an attempt to explain the biogenesis of Δ-5,7,9-estratrienol-3(β)-one-17 (I) must necessarily be speculative, its derivation from estrone (VI) by the sequence of oxidation and reduction indicated is in accord with the known facts. Equilenin (III),
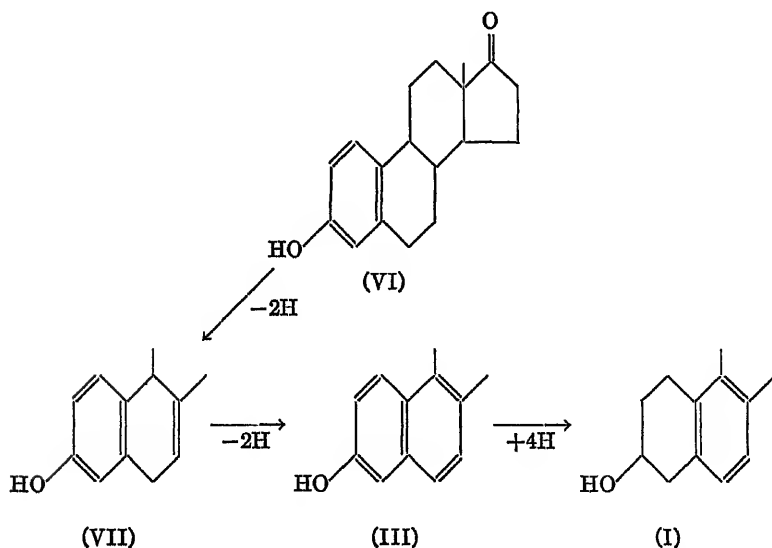
excreted in relatively large amounts toward the end of gestation in the mare (18), is generally regarded (*vide* Fieser (13)) as arising from estrone (VI) by dehydrogenation. In support of this concept is the occurrence in urine of smaller quantities of phenolic estrogens containing an ethylenic linkage in Ring B, which may be considered as intermediary compounds in the aromatization process, *i.e.*, equilin (VII), the output of which also rises as parturition approaches (19), hippulin (20), and a diol possessing the double bond in conjugation to the benzenoid ring, recently isolated by Hirschmann and Wintersteiner (21) and believed to be a Δ_{6-7}-17-dihydroequilin (22). The fact that, *in vitro*, Ring A of equilenin (III) can readily be saturated, whereas Ring B is difficult to reduce (6-8), makes reasonable the supposition that the same selective hydrogenation (III to I) takes place *in vivo*.

<div style="text-align:center">EXPERIMENTAL</div>

Melting points were taken on a long stem thermometer with the aid of a low power microscope; the values recorded are uncorrected.

Isolation from Urine of Δ-5,7,9-Estratrienol-3(β)-One-17 (I) As Acetate and Benzoate—124 gm. of hydroxy ketonic oil, separated as previously described (2) from the non-phenolic extract of equine pregnancy urine (approximately 10,000 gallons),[4] were fractionated *in vacuo* (about 0.1 mm.) at the following oven temperatures, up to 115° (63.1 gm.), from 115–140° (14.6 gm.), from 140-170° (9.6 gm.), and from 170–210° (18.5 gm.). To the distillate collected from 170- 210°, in ethanol (150 ml.), were added 3 liters of a hot ethanolic solution of digitonin (1 per cent). The insoluble digitonides[5] precipitated after 24 hours in the refrigerator were removed and washed with cold ethanol. The volume of the combined filtrate and washings was reduced to 100 ml., when ether (1 liter) was added, and the precipitated digitonin filtered off and washed with ether. On evaporation of the solvents, 11.5 gm. of

[4] We are indebted to E. R. Squibb and Sons for non-phenolic extract of equine pregnancy urine, and to Professor G. F. Marrian and Dr. A. D. Odell for the isolated ketonic fraction of a large batch of this material.

[5] Decomposition of the insoluble digitonides yielded a gum from which further quantities of the previously described androstanol-3(β)-one (2) were obtained.

digitonin-non-precipitable oil remained. This was taken up in 50 ml. of ethanol and refluxed for 2 hours with 20 gm. of semi-carbazide hydrochloride and 20.7 gm. of sodium acetate in 10 ml. of water. After 24 hours in the cold, the first crop of semicarbazones (A) was collected, washed with chilled ethanol and boiling water, and dried (593 mg., decomposing at 220–225°). The filtrate and ethanolic washings yielded a second crop (Semicarbazones B) on concentration (2 gm., decomposing at 200–213°). Both Semicarbazones A and B were amorphous materials; trials showed that repeated recrystallization from ethanol was ineffective as a means of purification. Evaporation of the mother liquors from Semicarbazones B gave a gum (11.7 gm.; Semicarbazones C) from which no solid could be separated.

Semicarbazones A (593 mg.) were hydrolyzed by refluxing for 75 minutes in 15 ml. of ethanol containing 7.5 ml. of 5 N sulfuric acid. The solution was diluted with 10 volumes of water and extracted three times with ether. After the combined ethers were washed with N sodium hydroxide solution and water, the solvent was distilled to give 443 mg. of an orange oil which failed to crystallize. It was therefore acetylated in pyridine (2 ml.) with 2 ml. of acetic anhydride (4 days at room temperature). The oil precipitated on addition of water was shaken out three times with ether, and the ethereal extracts were washed free of pyridine with 2 N hydrochloric acid, washed with N alkali and water, and taken to dryness. The residue, a bright orange oil, gave colorless crystals on distillation (150–160° and approximately 0.01 mm.). After two sublimations and three recrystallizations from aqueous ethanol and aqueous acetone, the melting point of the acetate of (I) was constant at 158° (30 mg. of rectangular plates).

$$\textit{Analysis} — C_{18}H_{21}O(OCOCH_3).$$

Calculated.	C 76 88,	H 7 75
Found.	" 76.98,	" 7.37

91.4 mg. of less pure acetate (m.p. 140–157°) were recovered from the mother liquors of the above crystallizations.

Semicarbazones B (2 gm.) were hydrolyzed in the same manner. In this instance the oily product (1.672 gm.), in pyridine (20 ml.), was treated with benzoyl chloride (5 ml.) for 24 hours at room temperature, and then worked up as described for the acetate.

A semicrystalline mass was obtained, which, after three recrystallizations from benzene-ligroin, yielded 404 mg. of crude benzoate in the form of rosettes of stout needles, sparingly soluble in ethanol (m.p. 185–193°).

An additional quantity of the benzoate was obtained from Semicarbazones C by chromatographic adsorption. The gum (11.7 gm.) was hydrolyzed to the free hydroxy ketones (6.712 gm.) which, on benzoylation, gave a non-crystallizable oil. The latter was dissolved in benzene (6 ml.), brought almost to the point of precipitation by addition of ligroin (54 ml.), and put through a column (90 × 2.2 cm.) of aluminium oxide (Merck, standardized

TABLE I

Chromatographic Separation of Benzoate of Δ-5,7,9-Estratrienol-3(β)-One-17

Fraction No.	Eluent (benzene-ligroin mixtures)	Eluate		
		Yield	Character	
		gm.		
1	100% ligroin	0		
2	10% benzene	0.425	Oil	
3	10% "	1.282	"	
4	10% "	1.755	"	
5	10% "	0.463	"	
6	20% "	0.826	"	
7	20% "	0.453	" and crystals	
8	20% "	0.251	" " "	
9	30% "	0.471	"	
10–12	30% "	0.844	"	

according to Brockmann; 300 gm.) which had been previously saturated with ligroin. Elutions were made with successive 300 ml. portions of benzene-ligroin mixtures, as indicated in Table I. Recrystallization of Fractions 7 and 8 from benzene-ligroin yielded respectively 113 and 20 mg. of benzoate, m.p. 196 198°, after preliminary softening at 190°.

Δ-5,7,9-Estratrienol-3(β)-One-17 (I)—The crude benzoate (537 mg.) was saponified by refluxing in 21 ml. of ethanolic potassium hydroxide solution (4 per cent) for 45 minutes, during which time a marked red coloration developed in the solution. The product was precipitated with water and extracted with ether. On

evaporation of the washed ethereal extracts, a red oil was obtained which was distilled at 160–165° and 0.01 mm.; the condensate consisted of colorless crystals, a highly pigmented residue remaining undistilled. The former was recrystallized from benzene-ligroin and again from aqueous ethanol to give a mixture of needles and monoclinic prisms (m.p. 133–134°; remelt unchanged). After another sublimation, four recrystallizations from aqueous ethanol failed to sharpen the melting point, but on each occasion it was raised approximately 1°. The final ethanolic solution, seeded with a crystal of the prismatic modification, gave up 45 mg. (analytical sample) of Δ-5,7,9-estratrienol-3(β)-one-17 entirely in this form (Fig. 1). An additional 87.5 mg. (m.p. 136–137°) were recovered from the mother liquors.

The crude acetate (91.4 mg.), saponified and worked up in the same way, yielded, after one sublimation and two recrystallizations, 30 mg. of prismatic crystals identical (mixed melting point) with those from the benzoate.

$$Analysis\text{---}C_{18}H_{22}O_2. \quad \text{Calculated.} \quad \text{C 79 95, H 8.20}$$
$$\text{Found.} \qquad \text{`` 80 08, `` 8.29}$$
$$[\alpha]_D^{27} = +59° \pm 3° \ (1.185\% \text{ in ethanol})$$

The ultraviolet absorption spectrum[6] (Curve A, Fig. 2) exhibits maxima at 269.5 and 278 mμ, with $\epsilon = 345$ and 240 respectively, and minima at 249 and 276 mμ, with $\epsilon = 95$ and 232.

Δ-5,7,9-Estratrienol-3(β)-one-17 is not precipitated with digitonin. It melts to a colorless oil, which, on being heated to approximately 240°, develops a bright red color, similar to but less intense than "equilenin red" (18). The Liebermann-Burchard and Salkowski reactions were strongly positive, the latter being reversed (like ergosterol); the Rosenheim test was negative. With tetranitromethane, a deep yellow coloration was produced, and with nitric acid in the cold, an orange-yellow color which was not altered on addition of ammonia. The red-violet color with the Zimmermann (9) ketone reagents developed at the same rate as with estrone and equilenin, but less rapidly than with pregnanedione.

Bromine titration was carried out by the Rosenmund-Kuhnhenn

method (23).   10.1 mg. of (I), in chloroform (2 ml.), with 5 ml. of the pyridine sulfate dibromide reagent, absorbed, after 10 minutes in the dark, 0.33 mg. of bromine (0.06 ethenoid linkage).

The *oxime*, prepared by refluxing (2 hours) 20 mg. of (I) in ethanol (1.5 ml.) with hydroxylamine (30 mg.) and sodium acetate (40 mg.) in water (1 ml.), had, after three recrystallizations from aqueous ethanol, a melting point of 195–197° with decomposition (16 mg. of fine needles).

$$Analysis—C_{18}H_{23}O_2N. \quad \text{Calculated} \quad C\ 75.74,\ H\ 8\ 13,\ N\ 4.91$$
$$\text{Found.} \quad "\ 75.77,\ "\ 8.52,\ "\ 4.70$$

Δ-5,7,9-Estratrienediol-3(β),17(α) *(II)*—To 15 mg. of previously reduced platinum oxide catalyst (Adams), suspended in ethanol (1 ml.), were added 29.35 mg. of (I) in 3 ml. of the same solvent.   The mixture was shaken in an atmosphere of hydrogen at room temperature and pressure until uptake of hydrogen ceased (80 minutes); 2.61 ml. (0.99 mole) were used.   The platinum was removed, and the residue from the filtrate and washings sublimed at 150–160° and 0.01 mm.   Plates collected in the proximal portion of the side arm of the distillation flask; these (8.3 mg.) had a melting point of 168–168.5° and were removed for analysis. The remainder of the distillate, a mixture of crystals and oil, gave, on crystallization from ethyl acetate, 15 mg. of slightly less pure material (m.p. 164–166°); mixed with Δ-5,7,9-estratrienediol-3(β),17(α) (m.p. 162–164°), prepared from equilenin[7] by hydrogenation in acid ethanol (method of Ruzicka, Müller, and Mörgeli (8)), the melting point was 163–165°.

$$Analysis—C_{18}H_{24}O_2. \quad \text{Calculated.} \quad C\ 79.35,\ H\ 8.88$$
$$\text{Found.} \quad "\ 79.43,\ "\ 8.80$$
$$[\alpha]_D^{23}\ (\text{specimen of m.p. 164–166°}) = -5° \pm 4°\ (0.945\%\ \text{in ethanol})$$

The diol is not precipitated with digitonin.[8]   When its melt is heated to 240°, no red color develops as with (I); the behavior

---

[7] Kindly furnished by Parke, Davis and Company, through the courtesy of Dr. Oliver Kamm.

[8] Hirschmann and Wintersteiner (21) have pointed out that the *trans* (α) configuration of the C_{17}—OH relative to the C_{13} methyl group is a "necessary but not sufficient condition for the formation of insoluble digitonides of this type;" precipitable are α-œstradiol (21) and androstanol-17(α) (unpublished observation of the authors), but not 17(α)-dihydroœquilenin (24), *trans*-testosterone (25), or Δ-5,7,9-estratrienol-17(α) (authors' observa-

in this respect is similar to that of 17-dihydroequilenin, which, in contrast to equilenin, does not turn red on prolonged heating (Hirschmann and Wintersteiner (21)).

The *diacetate* was prepared by treating (II) (11.6 mg.), in pyridine (8 drops), with acetic anhydride (10 drops) for 24 hours at room temperature. The ethereal extracts of the diluted solution were washed with 2 N hydrochloric acid, N alkali, and water, and taken to dryness. Sublimation of the residue at 110–115° and 0.01 mm., and two recrystallizations of the sublimate from aqueous ethanol, gave 6 mg. of platelets melting sharply at 115°, both alone and on admixture with an authentic specimen[9] of the diacetate of Δ-5,7,9-estratrienediol-3(β),17(α) (m.p. 115–116°).

Analysis—$C_{18}H_{22}(OCOCH_3)_2$. Calculated. C 74 12, H 7 92
                                        Found.   " 74.16, " 7.85

Δ-*3,5,7,9-Estratetraenone-17* (*IV*)—An intimate mixture of (I) (30.3 mg.) and freshly fused potassium hydrogen sulfate (80 mg.), in a Pyrex U-tube from which air was expelled by passage of a steady stream of pure nitrogen, was heated at 150–155° for 1 hour. The mass was then broken up in water, and the suspension extracted several times with ether. Distillation of the residue from the washed ethereal extracts at 130–140° and 0.01 mm. yielded a small quantity of colorless oil; a considerable portion, presumably consisting of polymeric products (*vide* Haslewood and Roe (4)), remained undistilled below 200°. Crystallization of the distillate from aqueous ethanol gave 3.5 mg. of slender white needles, m.p. 96–100°, which exhibited the absorption spectrum illustrated in Fig. 2 (Curve C). Three additional recrystallizations from the same solvent raised the melting point to 114–116°.

SUMMARY

From the urine of pregnant mares there has been isolated a new hydroxy ketone, $C_{18}H_{22}O_2$, m.p. 138–139.5°, $[\alpha]_D +59°$, which

---

tion). Apparently a similar irregularity obtains with 3(β)-hydroxy steroids in the absence of the C_{10} methyl group. Neither the urinary hydroxy ketone (I) nor the diol (II) is precipitated, although, judging from the physical evidence (see the introduction), the C_3—OH is β-oriented in each, whereas neoergosterol (26) and one of the hexahydrodesoxoestrones (27) form insoluble digitonides.

[9] Provided by Professor L. Ruzicka, to whom our thanks are expressed.

forms an acetate, m.p. 158°, a benzoate, m.p. 196-198°, and an oxime, m.p. 195-197°. Reduction of the carbonyl group yielded Δ-5,7,9-estratrienediol-3,17(α), identical with the lower melting C_3 epimer obtained on saturation of Ring A of 17(α)-dihydro-equilenin. The position of the hydroxyl group on C_3 was revealed by dehydration which introduced an ethylenic linkage in conjugation to the bonds of the aromatic Ring B. The compound is therefore Δ-5,7,9-estratrienol-3-one-17, a neutral isomer of estrone in which Ring B instead of Ring A is benzenoid. Evidence supporting the β orientation of the C_3—OH is set forth. It is suggested that the urinary steroid is derived in the body from equilenin.

BIBLIOGRAPHY

1. Heard, R. D. H., and Hoffman, M. M., *J. Biol. Chem.*, **135**, 801 (1940).
2. Heard, R. D. H., and McKay, A F., *J. Biol. Chem.*, **131**, 371 (1939).
3. Askew, F. A., *J Chem. Soc.*, 512 (1935).
4. Haslewood, G. A. D., and Roe, E., *J. Chem. Soc.*, 465 (1935).
5. Inhoffen, H. H , *Ann. Chem.*, **497**, 130 (1932).
6. Windaus, A., and Deppe, M., *Ber. chem. Ges.*, **70**, 76 (1937).
7. David, K., *Acta brev. Neerl.*, **8**, 211 (1938).
8. Ruzicka, L., Müller, P., and Morgeli, P., *Helv. chim. acta*, **21**, 1394 (1938).
9. Zimmermann, W., *Z. physiol. Chem.*, **233**, 257 (1935).
10. Callow, R. K., and Young, F. G., *Proc. Roy. Soc. London, Series A*, **157**, 194 (1936).
11. Cook, J. W., and Haslewood, G. A. D., *Chem. and Ind.*, **53**, 507 (1934).
12. Chakravorty, P. N., and Wallis, E. S., *J. Am. Chem. Soc.*, **60**, 1379 (1938).
13. Fieser, L. F., The chemistry of natural products related to phenanthrene, New York, 2nd edition (1937).
14. Marker, R. E., Rohrmann, E., Wittle, E. L., and Tendick, F. H., *J. Am. Chem. Soc.*, **60**, 2440 (1938).
15. Reindel, F., and Niederländer, K., *Ann. Chem.*, **522**, 218 (1936).
16. Remesov, I. A., *Rec. trav. chim. Pays-Bas*, **55**, 797 (1936); **56**, 1091 (1937).
17. Wintersteiner, O., and Smith, P. E., in Luck, J. M., Annual review of biochemistry, Stanford University, **7**, 253 (1938).
18. Sandulesco, G., Tchung, W. W., and Girard, A., *Compt. rend Acad.*, **196**, 137 (1933).

19. Girard, A., *Bull. Soc. chim biol.*, **15**, 562 (1933).
20 Girard, A , Sandulesco, G., Fridenson, A., Gaudefroy, C., and Rutgers, J. J., *Compt. rend. Acad.*, **194**, 1020 (1932).
21. Hirschmann, H., and Wintersteiner, O., *J. Biol Chem.*, **122**, 303 (1937–38).
22. Hirschmann, H., and Wintersteiner, O , *J Biol Chem.*, **126**, 737 (1938).
23. Rosenmund, K. W., and Kuhnhenn, W., *Z. Untersuch. Nahrungs-u. Genussmittel*, **46**, 154 (1923)
24. Marker, R. E., *J. Am. Chem. Soc.*, **60**, 1897 (1938).
25. Ruzicka, L , and Kägi, H., *Helv. chim. acta*, **19**, 842 (1936).
26. Bonstedt, K , *Z. physiol. Chem.*, **185**, 165 (1929).
27. Dirscherl, W., *Z. physiol. Chem.*, **239**, 53 (1936).

THE EFFECT OF A NICOTINIC ACID DEFICIENCY UPON THE COENZYME I CONTENT OF THE HUMAN ERYTHROCYTE AND MUSCLE*

By A. E. AXELROD, TOM D. SPIES, and C. A ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, and the Department of Internal Medicine, University of Cincinnati, Cincinnati)

The effect of a nicotinic acid deficiency upon the amount of coenzyme-like substances (designated as factor V in the microbiological methods) in various tissues of the dog has been studied by a number of workers. Kohn, Klein, and Dann (1) found decreases in the factor V concentrations in the liver and striated muscle of the dog. These results with the dog were confirmed by Pittman and Fraser (2) who, in addition, reported a decrease in the factor V content of the cardiac muscle of this species. Both of these groups of workers used *Haemophilus parainfluenzae* to measure factor V. Axelrod *et al.* (3), using a yeast fermentation method, found decreases in the coenzyme I content of liver and striated muscle of dogs and pigs suffering from a nicotinic acid deficiency.

Studies in man have confirmed the existence of a relationship between nicotinic acid and the pyridine nucleotides. Kohn (4) and Vilter, Vilter, and Spies (5) have observed an increase in the factor V content of blood after the administration of nicotinic acid, and Kohn and Klein (6) and Vilter, Vilter, and Spies (7) have shown that incubation of defibrinated blood with nicotinic acid results in an increase in the factor V content. In addition Axel-

rod, Gordon, and Elvehjem (8) have demonstrated that the ingestion of large amounts of nicotinic acid is followed by an increase in the coenzyme I content of erythrocytes.

Vilter *et al.* (5, 9), using *Bacillus influenzae* in a microbiological method, reported decreases in the amount of factor V in the whole blood of pellagrins.   On the other hand Kohn and Bernheim (10), using *Haemophilus parainfluenzae*, did not find any significant decreases in the factor V of the erythrocytes from the blood of pellagrins.   The present report is concerned with a study of the erythrocyte and muscle coenzyme I values in a series of normal and pellagrous subjects and with the alterations in coenzyme I values induced by the administration of nicotinic acid, pyrazinemonocarboxylic acid, and coramine.   In addition, the abilities of nicotinic acid amide, coramine, quinolinic acid, and pyrazinemonocarboxylic acid to effect an *in vitro* synthesis of coenzyme I in defibrinated blood from normal subjects were investigated.   A yeast fermentation method specific for the determination of coenzyme I was employed throughout (11).

Material and Methods

The clinical material was obtained from a large series of pellagrins who were admitted to the Nutrition Clinic, Hillman Hospital, Birmingham, Alabama.   The patients were classified arbitrarily according to the severity of their pellagrous condition in the following manner: +, mental or physical poor health without the classical lesions of pellagra; ++, mild classical lesions of pellagra; +++, moderately severe lesions of pellagra; ++++, severe lesions of pellagra.   In all cases, the determination of the coenzyme I content of erythrocytes and muscle was made before the initiation of any therapy.   The normal control subjects for the blood studies were chosen from members of the hospital and laboratory staffs.   Patients in the Hillman Hospital who were undergoing a herniorrhaphy were used as normal controls for the muscle studies.   Eight pellagrins were selected for a study of the effect of the administration of nicotinic acid, pyrazinemonocarboxylic acid, and coramine upon the coenzyme I content of erythrocytes and muscle.   In this group, coenzyme I determinations were made before and after the administration of the various antipellagric compounds as indicated in Table III.

The methods used for the coenzyme I determinations of both erythrocytes and muscle are fully described in a previous publication (12).[1] Blood was obtained by venipuncture and potassium oxalate was used as the anticoagulant. Hematocrit values were determined for all blood samples. A section of the quadriceps femoris muscle (approximating 2 gm.) was obtained by biopsy and a small portion was used for a moisture determination. The remainder of the muscle was immediately frozen on a slab of carbon dioxide and the extract prepared as previously described (12). The tissue extracts were stored in the refrigerator and were always analyzed within 24 hours after their preparation. All biopsy material was obtained under local anesthesia instituted by infiltration with novocaine down to and including the deep fascia. By this procedure the muscle obtained for study was not altered by novocaine. Recovery experiments with pure coenzyme I were carried out on a series of muscle extracts in order to determine whether the local anesthetic had any detrimental effect on the assay. The muscle extracts were found to contain nothing which inhibited or accelerated the yeast fermentation. The addition of 0.1 ml. of a 2 per cent solution of novocaine had no effect upon the recovery of coenzyme I.

The *in vitro*[2] experiments which were designed to study the abilities of nicotinic acid amide, coramine, quinolinic acid, and pyrazinemonocarboxylic acid to effect the synthesis of coenzyme I in defibrinated blood were carried out in the following manner. The compounds were prepared in isotonic saline at a concentration of 500 γ per ml. Each solution was brought to pH 7.4 and was sterilized by filtration through a Berkefeld filter. 1 ml. of each solution was used per ml. of whole blood. A control tube containing isotonic saline was employed in each set of experiments. Fresh, defibrinated blood from normal subjects was incubated at 37° under sterile conditions with each of the above compounds. After an incubation period of 24 hours, the coenzyme I contents of the erythrocytes were determined in the usual manner. The effect of each compound was studied with three different samples of blood.

Observations

Blood Studies before Therapy—Table I shows a range in the coenzyme I content of erythrocytes from 40 to 110 γ per ml. The normal subjects averaged 85 and the pellagrins of classification + and ++ averaged 77 and 69 γ of coenzyme I per ml. of erythrocytes respectively. These figures indicate no significant difference between average values for the different groups. The distribution of values given in Table I indicates that a moderate tendency toward lower values may be correlated with an increasing severity of the deficiency. Thus, the erythrocyte coenzyme I values of 22

TABLE I

Distribution of Coenzyme I Values of Erythrocytes from Normal and Pellagrous Subjects

	Coenzyme I per ml. of erythrocytes							Average values of coenzyme I per ml. of erythrocytes	Standard deviation
	40-50 γ	50-60 γ	60-70 γ	70-80 γ	80-90 γ	90-100 γ	100-110 γ		
	No. of cases in each range of values								
								γ	γ
Normal	0	3	7	12	10	10	3	85	15
+	2	2	6	7	10	2	2	77	13
++	3	3	2	4	2	3	0	69	17
+++	0	0	0	1	2	0	0		
++++	0	0	0	1	1	0	0		

per cent of the normal subjects, 32 per cent of the + group, and 47 per cent of the ++ group fell below 70 γ per ml. It is obvious that the determination of the coenzyme I content of erythrocytes, by means of the yeast fermentation method, would be of little value as a diagnostic test for pellagra.

Although practically all of the coenzyme I in blood is confined to the erythrocytes, it is questionable whether the expression of the coenzyme I per ml. of packed cells is a more valid unit of concentration than that based upon the coenzyme I content per ml. of whole blood. Vilter and coworkers (9) have discussed the various aspects of this problem, and their views are consistent with the work of Melnick, Robinson, and Field (13), who have reported that the concentration of nicotinic acid in the corpuscles may vary with the hematocrit values.

When we calculated our results on the basis of the coenzyme I content per ml. of whole blood, we found that the resulting values paralleled those obtained when the coenzyme I values were expressed per ml. of erythrocytes.   Thus, the average value for the normal group was 34 γ of coenzyme I per ml. of whole blood, that of the + group was 30 γ per ml., and the average value for the more severe pellagrins was 26 γ of coenzyme I per ml. of whole

TABLE II

Effect of Varying Degrees of Nicotinic Acid Deficiency upon Coenzyme I Content of Human Muscle

The values are expressed as micrograms of coenzyme I per gm. of fresh muscle.

Normal	+	++	+++	++++
411	275	200	246	78
360	370	257	296	267
378	347	257	183	
360	295	293		
424	362	229		
390	293	273		
352	343	283		
377	236	199		
388	380	314		
	278	252		
	308	256		
		260		
		250		
		283		
Average . . 382	317	258	242	
Standard deviation 22	44	31		

blood.   The differences between these average values are not significant.

Muscle Studies before Therapy—Table II shows a range in the coenzyme I content of muscle from 78 to 424 γ per gm. of fresh muscle.   The normal subjects averaged 382 γ per gm., while the average values for the +, ++, and +++ groups were 317, 258, and 242 γ of coenzyme I per gm. of fresh muscle respectively.   It is noticeable that the coenzyme I content of muscle decreased

as the deficiency became more severe. The variations from the normal values first became significant in the $++$ group. No

TABLE III

Changes in Coenzyme I Content of Human Erythrocytes and Muscle Following Administration of Nicotinic Acid and Other Antipellagric Compounds

Case No.	Classifi-cation	Compound administered	Period of treat-ment	Coenzyme I content of			
				Erythrocytes		Muscle	
				Before treat-ment	After treat-ment	Before treat-ment	After treat-ment
			days	γ per cc. erythro-cytes	γ per cc. erythro-cytes	γ per gm. fresh muscle	γ per gm. fresh muscle
I	$++$	Coramine*	3	77	78	229	222
II	$++++$	Nicotinic acid†	5	78	136	267	262
III	$+++$	Quinine nicotinate‡	2	69	111	250	232
		Nicotinic acid	7	111	196	232	326
IV	$++$	" "	6	76	185	200	304
V	$++++$	Pyrazinemonocarbox-ylic acid§	3	82	90	78	138
		Nicotinic acid	3	90	124	138	187
		" "	5	124	185	187	223
VI	$+++$	Pyrazinemonocarbox-ylic acid	3	82	119	206	202
		Nicotinic acid	2	119	158	202	278
VII	$+++$	" "	7	85	182	183	314
VIII	$++$	Pyrazinemonocarbox-ylic acid	4	85	88	293	248
		Nicotinic acid	8	88	190	248	328

* A daily dose of 24 ml. of a 25 per cent solution was administered orally.

† 1 gm. of nicotinic acid (orally) and 50 mg. of nicotinic acid (intravenously) were given daily.

‡ Quinine nicotinate equivalent to 450 mg. of nicotinic acid was given daily by mouth.

§ Pyrazinemonocarboxylic acid was given in the same dosage and by the same methods of administration as was nicotinic acid.

significant variations were found in the moisture content of muscle from the various groups.

Effect of Administration of Various Antipellagric Compounds upon Coenzyme I Content of Blood and Muscle—The results of the studies on the relationship of nicotinic acid, pyrazinemonocar-

boxylic acid, and coramine therapy to the coenzyme I content of erythrocytes and muscle are given in Table III.   It is evident that the administration of nicotinic acid caused a marked increase in the coenzyme I content of both erythrocytes and muscle.   The one patient (Case II) whose muscle coenzyme I content was not affected by nicotinic acid therapy was suffering from an infected biopsy wound and had a temperature of 38.8° during the period of nicotinic acid administration.   However, the coenzyme I content of the erythrocytes of this patient was increased.   In only one patient was the administration of pyrazinemonocarboxylic acid followed by an increase in the coenzyme I content of the erythrocytes and a similar increase in muscle was noted in only one case. No increase in the coenzyme I content of either erythrocytes or muscle was observed following coramine therapy.

In Vitro Studies—The *in vitro* studies with defibrinated blood demonstrated that the synthesis of coenzyme I could be accomplished only in the presence of nicotinic acid amide, when an increase of 60 per cent in the coenzyme I content was observed. Incubation of defibrinated blood with pyrazinemonocarboxylic acid, quinolinic acid, and coramine did not result in the synthesis of coenzyme I.

DISCUSSION

The results of the studies in man reported in this paper have confirmed previous observations made with the dog and pig (3). It is now evident that a nicotinic acid deficiency in these three species has but slight effect upon the coenzyme I content of the erythrocytes but that it causes a marked decrease in the coenzyme I content of striated muscle.   In addition, decreases in the coenzyme I content of the livers of dogs and pigs have been observed (3).   A yeast fermentation method which is specific for coenzyme I was used in these experiments, and it should be emphasized that the results obtained have no bearing upon the coenzyme II content of the tissues.   On the other hand, factor V which is determined by microbiological methods involving the use of either *Haemophilus parainfluenzae* or *Bacillus influenzae* is a measure of both coenzyme I and coenzyme II and, possibly, other related substances.   Other workers, using the microbiological methods, have found decreases in the factor V concentrations of liver, striated muscle, and cardiac muscle of dogs suffering from a nico-

tinic acid deficiency (1, 2). No decreases in the factor V content of dog erythrocytes were observed. Thus, in the dog, both the factor V and coenzyme I contents of erythrocytes, liver, and muscle seem to be affected in the same manner by a nicotinic acid deficiency.

In man the situation is complicated by the fact that somewhat different results are obtained when *Haemophilus parainfluenzae* and *Bacillus influenzae* are used to measure factor V. With regard to the coenzyme changes in the blood of pellagrins our results with the yeast fermentation method are in better agreement with those obtained by Kohn and Bernheim (10) using *Haemophilus parainfluenzae* than with the results of Vilter *et al.* (5) who employed *Bacillus influenzae*. In a later paper Vilter and coworkers (9), studying a group of pellagrins similar to that of the present study, have reported values for the factor V content of erythrocytes which are in closer agreement with the coenzyme I values given in the present paper.

The precise physiological significance of the lowering in the coenzyme I content of muscle cannot be evaluated until more is known about the quantitative relationship between the coenzyme I content of human muscle and the ability of the muscle to carry out its normal oxidative functions. A decrease in the factor V level of dog muscle is accompanied by changes in the oxidative metabolism of the muscle (1) and we may expect to find a similar relationship in the case of human muscle.

The ineffectiveness of pyrazinemonocarboxylic acid and quinolinic acid in the prevention or cure of blacktongue in the dog has been demonstrated by Dann *et al.* (14) and Waisman and associates (15). Using *Haemophilus parainfluenzae*, Dann *et al.* (14) found that neither of the above compounds could effect an *in vivo* or an *in vitro* synthesis of factor V in human blood. Bills and coworkers (16) and Vilter and Spies (17) have shown that both pyrazinemonocarboxylic acid and quinolinic acid are effective in the cure of pellagra and in contrast to Dann *et al.* (14) have found, with *Bacillus influenzae*, that the administration of these compounds caused an increase in the factor V content of human blood. Smith, Ruffin, and Smith (18) have reported that coramine is approximately one-fourteenth as effective as nicotinic acid in the cure of experimental blacktongue and one-seventh as effective as

nicotinic acid in the cure of pellagra. Our results indicate that the antipellagric value of a compound is not necessarily associated with its ability to affect the coenzyme I content of tissues. Thus nicotinic acid has a marked effect upon the coenzyme I content of human blood both *in vivo* and *in vitro*. In this respect our findings parallel those obtained by other workers who have studied the relationship of nicotinic acid to the factor V content of human blood with microbiological methods (4–7). The increase in the coenzyme I content of human muscle following nicotinic acid therapy lends further support to the hypothesis that the antipellagric value of a compound depends upon its ability to influence the coenzyme content of tissues. On the other hand, our results with pyrazinemonocarboxylic acid and coramine do not support such a conclusion. Treatment with these compounds resulted in a definite clinical improvement in the pellagrins which was not accompanied by consistent changes in the coenzyme I content of erythrocytes or muscle.

SUMMARY

1. The coenzyme I content of erythrocytes does not decrease significantly in varying stages of pellagra. In forty-five control subjects the average value was 85 γ of coenzyme I per ml. of erythrocytes; in thirty-one subclinical pellagrins the average value was 77 γ per ml., while in seventeen mild pellagrins the average value was 69 γ of coenzyme I per ml. of erythrocytes. Values ranging between 70 and 90 γ of coenzyme I per ml. of erythrocytes were found in five severe pellagrins, three of whom were ambulatory.

2. The coenzyme I content of striated muscle was determined in a series of normal subjects and patients with varying degrees of pellagra. In nine normal subjects, the average value was 382 γ per gm. of fresh muscle, in eleven subclinical pellagrins the average value was 317 γ per gm., and in fourteen mild pellagrins the average value was 258 γ per gm. of fresh muscle. The average coenzyme I content of the muscles from five severe pellagrins was 214 γ per gm. In general, the coenzyme I content of muscle decreases as the deficiency becomes more severe.

3. The administration of nicotinic acid to pellagrins led to marked increases in the coenzyme I content of human erythrocytes and muscle. Coramine therapy, studied in one patient, had no

effect upon the coenzyme I content of either muscle or erythrocytes. Pyrazinemonocarboxylic acid when administered to three patients caused an increase in the coenzyme I content of the erythrocytes of one patient and an increase in the muscle coenzyme I content of another. All three of these compounds, however, caused a definite improvement in the clinical condition of the pellagrins.

4. Of the four compounds tested (pyrazinemonocarboxylic acid, quinolinic acid, coramine, and nicotinic acid amide) only nicotinic acid amide effected an *in vitro* synthesis of coenzyme I in defibrinated blood.

BIBLIOGRAPHY

1. Kohn, H. I., Klein, J. R , and Dann, W. J., *Biochem. J.*, **33**, 1432 (1939).
2. Pittman, M., and Fraser, H. F., *Pub. Health Rep., U. S. P. H. S.*, **55**, 915 (1940).
3. Axelrod, A. E., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 85 (1939).
4. Kohn, H I., *Biochem. J.*, **32**, 2075 (1938).
5 Vilter, R. W , Vilter, S. P., and Spies, T. D., *J. Am. Med. Assn.*, **112**, 420 (1939).
6. Kohn, H. I., and Klein, J. R., *J. Biol. Chem.*, **130**, 1 (1939).
7. Vilter, S. P., Vilter, R. W., and Spies, T. D., *Nature*, **144**, 943 (1939).
8. Axelrod, A. E., Gordon, E. S., and Elvehjem, C. A., *Am. J. Med. Sc.*, **199**, •697 (1940).
9. Vilter, S. P., Koch, M. B., and Spies, T. D., *J. Lab. and Clin. Med.*, **26**, 31 (1940).
10. Kohn, H. I., and Bernheim, F., *J. Clin. Inv.*, **18**, 585 (1939).
11. von Euler, H., *Ergebn. Physiol.*, **38**, 1 (1936).
12. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 77 (1939).
13. Melnick, D., Robinson, W. D., and Field, H., Jr., *J. Biol. Chem.*, **136**, 157 (1940).
14. Dann, W. J., Kohn, H. I., and Handler, P., *Proc. Am. Inst. Nutrition, J. Nutrition*, **19**, 8 proc. (1940).
15. Waisman, H. A., Mickelson, O., McKibbin, J. M., and Elvehjem, C. A., *J. Nutrition*, **19**, 483 (1940).
16. Bills, C. E., McDonald, F. G., and Spies, T. D., *Southern Med. J.*, **32**, 793 (1939).
17. Vilter, R. W., and Spies, T. D., *Lancet*, **2**, 423 (1939).
18. Smith, D. T., Ruffin, J. M., and Smith, S. G., *Proc. Am. Inst. Nutrition, J. Nutrition*, **19**, 14 proc. (1940).

THE GLUTAMIC ACID OF MALIGNANT TISSUE PROTEINS

By GLADYS E. WOODWARD, FRANCIS E. REINHART, AND
JANETTA SCHOONOVER DOHAN

*(From The Biochemical Research Foundation of the Franklin Institute,
Newark, Delaware)*

On the basis of the observed optical activity of amino acids isolated from hydrolysates, Kögl and Erxleben (1–4) claimed, in 1939, that the proteins of malignant tissues differ from those of normal tissues in that several of the constituent amino acids, especially glutamic, exist in a partially racemized state, while only the "natural" or *l* forms occur in normal tissues. Because of the importance of this claim many investigators attempted to repeat the experimental findings of these authors, especially with respect to glutamic acid. Although some samples of this acid showing a considerable degree of racemization (5–9) were obtained from malignant tissues, the majority of the samples isolated showed very little or no racemization (8–15).

Recently Chibnall and coworkers (13) produced evidence which offered an explanation for these anomalous results. Kögl assumed that the glutamic acid remaining in the hydrolysate was equally or even more highly racemized than the isolated portion of the total acid. This assumption was based on the observation (2) that racemic glutamic acid hydrochloride was more soluble in hydrochloric acid than the *l* form. Chibnall showed that this solubility relationship was not necessarily the same in protein hydrolysates and that it was possible, at least under the conditions of the cuprous oxide procedure used by Kögl, to precipitate preferentially a small fraction of the total glutamic acid which contained all or most of the racemic form. The degree of racemization of an isolated sample could have been dependent, therefore, on the yield.

In order to have a more representative value for comparison of different tissues Chibnall calculated in terms of total weight of

dry tissue the percentage of $d(-)$ acid obtained.   On this basis the $d(-)$-glutamic acid values for a number of normal as well as malignant tissues, regardless of the analytical procedure, corresponded to about 0.2 per cent of the total protein.   When the data of Kögl were calculated in this way, six out of nine malignant tissues were shown to have yielded similar small amounts of $d(-)$-glutamic acid.   Kögl's claim that the glutamic acid of tumors is highly racemized appears, therefore, to be based largely on an incorrect interpretation of data.   Additional evidence that both normal and malignant tissues yield amounts of the $d(-)$ acid to the extent of only a few tenths of 1 per cent was presented by Johnson (14) who also used an isolation procedure.   Similar results were obtained by Graff et al. (15) and by Behrens et al. (16) using isotopic methods of estimation which are claimed to account quantitatively for the glutamic acid present.   Since it had been shown by Johnson and by Behrens that $l(+)$-glutamic acid became racemized during prolonged boiling in hydrochloric acid to an extent which could account for the presence of these small amounts of the $d(-)$ form, it was suggested by them that the $d(-)$ form may not have been present in the original tissue, but rather had been formed by racemization during hydrolysis.

Soon after publication of Kögl's original report, an investigation of the stereochemical nature of glutamic acid in malignant tissue proteins was begun in this laboratory.   Following as closely as possible either the Dakin or the cuprous oxide procedure as used by Kögl, we have isolated glutamic acid hydrochloride from six tumors, all but one of which were shown by pathological examination to be malignant.   In one case separate isolations by each method were made from the more necrotic and less necrotic portions, since Dittmar (8) indicated that greater amounts of the $d(-)$ form might be present in the necrotic areas.   In another case, the hydrochloride was isolated from the soluble and the insoluble protein fractions by the cuprous oxide procedure.   In the remaining cases material representative of the whole tumor was used.

After much of our isolation work had been done, it became desirable to find a method for direct estimation of glutamic acid in protein hydrolysates, so that the efficiency of the isolation procedures might be ascertained.   Control experiments showed that

the method of Cohen (17), which involves conversion of glutamic to succinic acid and enzymatic determination of the latter by succinoxidase, could be adapted to such solutions. In this way the glutamic acid was determined in the tumor hydrolysates either before or after application of the isolation procedures.

The results obtained from the isolation of glutamic acid from the various tissues are summarized in Table I. In five cases (Nos. 2, 5-b, 5-d, 6-a, 6-b) the amounts of purified hydrochloride were represented, as in Kögl's work, by a single sample. In the remaining experiments additional fractions were recovered from the mother liquors obtained during the purification and, in two instances (Cases 5-a, 5-c), a third fraction from the propyl alcohol extract used in the course of the Dakin procedure. The purity of all fractions was established by C, H, N, and ash analyses. Difficulty in obtaining ash-free samples was encountered in certain experiments. In these cases, however, the ash content was below that which is detectable by nitrogen analysis, a criterion for purity used by the majority of workers in this field, and below that which would affect the specific rotation by an amount greater than the error involved in the determination of the latter. Where the ash content did exceed 1 per cent, correction of analytical and optical rotation data has been made.

As can be seen from Column 10 (Table I), we have been unable to confirm[1] the findings of Kögl that a significant percentage (6 to 45 per cent) of the glutamic acid isolated from malignant tissue is the $d(-)$ form. In no case did the specific optical rotation of any fraction of the purified glutamic acid hydrochloride, isolated from malignant as well as non-malignant tissue, indicate a $d(-)$ content greater than 4.4 per cent; the average $d(-)$ content of the total hydrochloride isolated from any one tissue was not over 2.5 per cent, a value which, as indicated by Kögl, is of questionable significance. When the $d(-)$ content is based on the weight of dry tissue used, it varies, as may be seen in Column

[1] A preliminary report from this laboratory (18) indicated that we had confirmed the findings of Kogl and Erxleben. The report was based on the observed low rotation of a sample of glutamic acid isolated from malignant tissue. More detailed analysis of the product involved showed that the low rotation was due largely to the presence of impurities rather than to $d(-)$-glutamic acid.

TABLE I

Glutamic Acid Isolated from Tumor Proteins

Case No.	Kind of tumor	Weight of dry protein	Method	Yield of glutamic HCl (purified)	Ash	Analyses			$[\alpha]_D$ in 9 per cent HCl, l-(+) form = +31.7°	d(−) form in isolated sample	d(−) form in total protein	Free glutamic acid (calculated)	Yield as per cent of protein	Glutamic acid content (Cohen)	Per cent of total acid isolated
						C, theory 32.71	H, theory 5.49	N, theory 7.63							
(1)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
		gm.		mg.	per cent	per cent	per cent	per cent	degrees	per cent	per cent	mg.	per cent	per cent	per cent
1	Flexner-Jobling carcinoma	16	Dakin	603	0.3	32.39	5.33	7.78	+30.7	1.6	0.05	483			
				175	0.2	32.98	5.43	7.66	+31.1	0.9	0.01	140			
				140	0.4	32.57	5.26	7.58	+29.2	3.9	0.03	112			
				83	1.3	32.24	5.55	7.83	+29.3	3.8	0.02	67			
				1001						(2.0)*	0.11	802	5.0	7 5	67
2	Jensen sarcoma	22	"	1043	0.3	32.57	5.74	7 76	+31.1	0.9	0.03	836	3.8		
3	Breast adenoma	22	Cuprous oxide	986	0.4	32 81	5.48	7.61	+30.2	2.4	0.09	790			
				39	1.1	32.71	5.60	7.77	+29.2	3.9	0.01	31			
				1025						(2.5)*	0.10	821	3.7	7.6	49
4	Lung carcinoma	27	Dakin	1542	1.5	32.46	5.79	7.70	+30.3	2 2	0.10	1235			
				119	1.9	32.88	5.66	7.53	+29.8	3.0	0.01	95			
				1661						(2.3)*	0.11	1330	4.9	7.5	65

5-a	Adrenal para-ganglioma, necrotic	20	Dakin	857	0.2	32.67	5.54	7.75	+31.0	1.1	0.04	687	4.2	6.0	70
				94	1.1	32.80	5.49	7.77	+28.9	4.4	0.02	75			
				108	1.5	32.88	5.66	7.77	+29 6	3.3	0.01	87			
				1059						(1.6)*	0.07	849			
5-b	Same	10	Cuprous oxide	504	0.0	32.69	5.53	7.71	+30.6	1.7	0.07	404	4.0	7.8	52
5-c	" less necrotic	20	Dakin	739	0.0	32.68	5.58	7.57	+31.6	0.0	0.00	592	4 6	9 5	48
				186	0 0	32.43	5 75	7.68	+29.5	3.5	0.03	149			
				218	0.0	32 69	5.60	7 80	+30.6	1.7	0.01	175			
				1143						(0 9)*	0.04	916			
5-d	Same	7.63	Cuprous oxide	535	0 0	32.58	5.66	7 79	+32.0	0	0	429	5.6	9.5	59
6-a	Abdominal carcinoma, insoluble part	6.3	" "	371	0.0	32 69	5.75	7.81	+31.3	0.6	0.03	297	4.7	10.1	47
6-b	Same, soluble part	5 85	" "	477	2.1	32 45	5.67	7 72	+30.3	2.2	0 14	382	6.5	12.0	55

* Calculated to express the $d(-)$ content of the total hydrochloride isolated.

11, from 0 to 0.14 per cent, which is of the same order of magnitude as that found by Chibnall and by others (14–16) for either malignant or normal tissue. There was no significant difference between the values found by us for the necrotic and non-necrotic parts of the tissue, nor for the soluble and insoluble protein; likewise, there was no significant difference between the results obtained by the cuprous oxide and the Dakin procedures.

The yield of purified acid, based on the weight of dry tissue, varied from 3.7 to 6.5 per cent (Column 13). That these values represent only a fair recovery is evident from a consideration of the results obtained by application of the Cohen procedure (Columns 14 and 15). Thus, the glutamic acid content of the tissues in Cases 1 to 5-b ranges between 6.0 and 7.8 per cent, so that the isolated sample represents 49 to 70 per cent of the acid present. These contents, however, are undoubtedly low, since they were obtained from the sum of the amounts isolated and the amounts present in the hydrolysates and mother liquors as estimated by the Cohen analysis. Any acid that might have been lost during isolation is therefore neglected. For the same reason the recoveries are correspondingly high. In Cases 5-c to 6-b, however, Cohen determinations were performed on aliquot samples of the tumor hydrolysates before isolation of the acid. The glutamic acid content in these cases is 9.5 to 12.0 per cent, values which lie in the same range as those found by Chibnall (modified Foreman method) and by Graff (isotope dilution method). The corresponding recoveries amount to 47 to 59 per cent.

If, as seems probable, the glutamic acid content of tumor tissues lies in the range of 8 to 12 per cent, the amounts isolated by Kögl in the majority of cases represented only a small proportion (less than 10 per cent) of the total acid present. Our results, on the other hand, indicate isolation of at least 47 per cent of the total acid. In spite of the fact that the optical rotation of the glutamic acid isolated in these higher yields would be expected to be more representative of that of the total acid than in the low yields of Kögl, our samples showed no appreciable racemization. Therefore, the evidence obtained by us does not support the claim that the glutamic acid of malignant tissue exists in a highly racemized state.

Adaptation of Cohen Procedure for Glutamic Acid (17) to Protein Hydrolysates

After hydrolysis of the tissue and partial clarification with cuprous oxide or charcoal, an aliquot of hydrolysate corresponding to not over 60 mg. of dry tissue is made to 3 cc. volume with water. After addition of a drop of brom-cresol green as indicator, the solution is neutralized to a bluish green (pH about 4.7) with approximately 2 N NaOH. If more than 10 drops are required, it is better to use a fresh sample, the neutralization being started with saturated NaOH, in order to keep the volume as low as possible. To this solution are added 1.5 cc. of 2 M citrate buffer, pH 4.7, and then 3 cc. of freshly dissolved 10 per cent chloramine-T. The mixture is placed in a bath at 40°, and shaken for 10 minutes. It is then cooled in ice, according to the Cohen procedure, and after 15 minutes filtered, being rinsed with a total of 4 cc. of cold water. With the volume now at about 11.5 cc., addition of 6.4 cc. of concentrated HCl brings the HCl concentration to slightly over 12.5 per cent as required. The rest of the procedure follows as described by Cohen.

Occasionally during the acid hydrolysis of the β-cyanopropionic acid the solution turns brown. This is due apparently to the presence of proline in the original tissue hydrolysate. The deep color does not interfere during the subsequent adjustment of the pH in the presence of phenol red, since it becomes much lighter just before the end-point is reached.

The extractors used in our work had a capacity of about 35 cc. Tests of their efficiency showed that the first extraction, in which p-toluenesulfonamide was removed, was complete within 2 hours; the second extraction, in which succinic acid was separated, was complete within 5 hours.

The possible interference by various amino acids which might be present in tissue hydrolysates was investigated. Alanine, arginine, aspartic acid, cysteine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, norleucine, phenylalanine, proline, serine, tyrosine, tryptophane, and valine carried through the procedure, both in the presence and absence of glutamic acid, gave no products which caused an oxygen uptake when heart was used as the source of succinoxidase. However, when

liver succinoxidase was used, serine was found to interfere (Dohan (19)). A product, probably glycolic acid, was formed which caused an oxygen uptake. Therefore, when the Cohen glutamic acid method is applied to tissue hydrolysates or any material which may contain serine, it is essential to use heart, as originally described, rather than liver succinoxidase.

The results of recovery experiments in which glutamic acid was added to an aliquot of tumor hydrolysate or to a mixture of amino acids (including serine) are reported in Table II. From these data it may be seen that the recovery values are of the same order as those found by Cohen for glutamic acid in pure solution or in the presence of protein.

TABLE II

Recovery of Glutamic Acid by Cohen Succinoxidase Method

Exper- iment No	Material to which added	Original glutamic acid content	Glutamic acid added	Total glutamic acid estimated	Glutamic acid recovered	Recov- ery
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	Tumor hydrolysate	6.45	7.35	13.82	7.37	100.3
2	" "	3.67	7.35	11.00	7.33	99.7
3	14 amino acids		10.47	9.98	9.98	95.3
4	18 " "		15.71	14.63	14.63	93.3

Isolation of Glutamic Acid As Hydrochloride

In all cases the procedures followed as closely as possible those used by Kögl. The tissues were finely minced and extracted with 0.6 per cent NaCl or water. The insoluble protein, as well as that precipitated by addition of 6 volumes of alcohol to the extract, was washed with 80 per cent alcohol, then with absolute alcohol, and dried at 80-90°. In one case, No. 6, the two fractions were analyzed separately but in all other cases they were combined. After hydrolysis with 3 parts of concentrated HCl for 7 hours, glutamic acid hydrochloride was isolated from the hydrolysates by the cuprous oxide or the Dakin procedure as used by Kögl. Recrystallization from 20 per cent HCl was carried out and, in several experiments, considerable amounts of glutamic acid hydrochloride were recovered from the mother liquors.

Case 1. Flexner-Jobling Rat Carcinoma—From 112 gm. of fresh tumors (3 weeks growth) 16 gm. of dry material were obtained. After the hydrolysate was treated by the Dakin butyl and propyl alcohol extraction procedure,· three fractions of crude glutamic acid hydrochloride, totaling 1557 mg., were obtained. The first fraction, weighing 1072 mg., gave a specific rotation of $[\alpha]_D = +25.0°$, but it was shown to contain considerable ash. The three fractions were worked up separately, recrystallizing from 20 per cent HCl, and gave finally the four pure products listed in Table I. Thus a total of 1001 mg. of pure glutamic acid hydrochloride was obtained. A Cohen glutamic acid analysis on the combined residues which consisted of the crude hydrolysate and recrystallization mother liquors showed that 392 mg. of glutamic acid had not been isolated.

Case 2. Jensen Rat Sarcoma—From 161 gm. of fresh tumors (3 to 4 weeks growth) 22 gm. of dry material were obtained. After the hydrolysate was extracted according to the Dakin procedure, 1764 mg. of crude glutamic acid hydrochloride were isolated. This sample was recrystallized twice by dissolving in dilute HCl and saturating with HCl gas at 0° to give 1118 mg. ($[\alpha]_D = +29.0°$, N 7.54 per cent, ash 2.9 per cent). A final recrystallization from 20 per cent HCl gave 1043 mg. of a pure product, as indicated in Table I. No analyses for glutamic acid by the Cohen method were carried out.

Case 3. Fibrocyst Adenoma of Breast (Non-Malignant, Human)—This tumor weighed 170 gm., from which 22 gm. of dry material were obtained. By means of the cuprous oxide procedure a single crop of crude glutamic acid hydrochloride (2200 mg.) was isolated. The sample contained a considerable amount of inorganic material. After the sample was recrystallized twice from 20 per cent HCl and the mother liquors worked up, a total of 1025 mg. of pure material, as indicated in Table I, was obtained. Glutamic acid analyses by the Cohen method showed that 810 mg. remained in the crude hydrolysate and only 49 mg. in the combined mother liquors obtained during the recrystallizations.

Case 4. Squamous Cell Carcinoma of Lung (Human)—The tumor was a solid encapsulated mass weighing 173 gm. which gave 27 gm. of dry material. After the hydrolysate was submitted to the Dakin extraction procedure, two fractions of crude glutamic

acid hydrochloride were isolated, 2417 mg., $[\alpha]_D = +25.7°$, and 394 mg. The latter was too impure for a rotation measurement. On recrystallization from 20 per cent HCl, followed by working up the mother liquors, two pure fractions, totaling 1661 mg., as indicated in Table I, were obtained. Cohen glutamic acid analyses on the residues showed that 558 mg. of glutamic acid were left in the crude hydrolysate and 149 mg. in the combined mother liquors from the recrystallizations.

Case 5. Paraganglioma of Adrenal Medulla (Human)—Since this was a very large tumor with considerable necrosis, it offered the opportunity of investigating separately the necrotic and non-necrotic material. That part which was highly necrotic, 356 gm., gave 68 gm. of dry material; 35 gm. were obtained from 265 gm. of the tissue which was but slightly necrotic. Each part was then worked up by both the cuprous oxide and Dakin procedures to give four analyses of this tumor, as follows:

(*a*) *Necrotic Section, Dakin Procedure*—The hydrolysate from 20 gm. of dry tissue yielded two fractions of crude glutamic acid hydrochloride. The first weighed 1128 mg., $[\alpha]_D = +25.9°$; the second, 251 mg., was too impure for a rotation measurement. Recrystallization from 20 per cent HCl until a low ash content was obtained and working up the mother liquors gave the first two fractions listed in Table I. The third fraction was obtained by recrystallization of the crude hydrochloride obtained when the propyl alcohol extract was reduced under a vacuum and the residue saturated with HCl. Cohen analysis on the mother liquors and crude hydrolysate showed that they contained 20 mg. and 325 mg. of the glutamic acid, respectively.

(*b*) *Necrotic Section, Cuprous Oxide Procedure* –From 10 gm. of dry tissue two crops of crude glutamic acid hydrochloride totaling 2445 mg. were obtained. This product was hygroscopic and undoubtedly very impure. Recrystallization from 20 per cent HCl gave 651 mg., $[\alpha]_D = +27.2°$. A second recrystallization yielded 504 mg. of the pure product listed in Table I. The total amount of glutamic acid left in the combined mother liquors and crude hydrolysate was shown by the Cohen analysis to be 380 mg.

(*c*) *Slightly Necrotic Section, Dakin Procedure*—The hydrolysate from 20 gm. of dry tissue yielded two crops of crude glutamic acid

hydrochloride totaling 1540 mg. Two recrystallizations and working up the mother liquors gave the first two pure fractions listed in Table I. The third fraction was obtained by recrystallization of a crude fraction isolated from the propyl alcohol extract as described under Case 5-a. No Cohen analyses were made on the residues, since the value for total glutamic acid in this tissue was determined in the following experiment.

(d) *Slightly Necrotic Section, Cuprous Oxide Procedure*—For the hydrolysis 10 gm. of dry tissue were used. From an aliquot of the hydrolysate corresponding to 7.63 gm. of dry tissue two crops of crude glutamic acid totaling 2203 mg. were obtained. One recrystallization from 20 per cent HCl gave 764 mg., $[\alpha]_D = +25.3°$; a second recrystallization yielded 535 mg., as listed in Table I. In this, and in the two subsequent cases, Nos. 6-a and 6-b, no attempt was made to recover glutamic acid from the mother liquors. Cohen analysis of an aliquot, taken from the original hydrolysate after clarification by cuprous oxide and subsequent removal of copper, showed that the latter contained an amount of glutamic acid equivalent to 9.5 per cent of the dry tissue used.

Case 6. Metastatic Abdominal Carcinoma, Primary in Thyroid (Human)—Insoluble and soluble protein fractions, weighing 28 gm. and 13 gm., respectively, were prepared from 500 gm. of fresh tumor tissue as described by Kögl and Erxleben, except that each fraction was washed thoroughly with ether before the final drying. The two fractions were analyzed separately by the cuprous oxide procedure, as follows:

(a) *Insoluble Fraction*—7 gm. of dry tissue were hydrolyzed and, after clarification by cuprous oxide, an aliquot of the hydrolysate equivalent to 6.3 gm. of tissue yielded 909 mg. of crude glutamic acid hydrochloride. One recrystallization from 20 per cent HCl gave 456 mg. ($[\alpha]_D = +29.5°$) which, after a second recrystallization, amounted to 371 mg., as indicated in Table I. Cohen analysis of an aliquot of the clarified hydrolysate showed that the total amount of glutamic acid present was equivalent to 10.1 per cent of the weight of dry tissue used.

(b) *Soluble Fraction*—From an aliquot of a hydrolysate prepared as in Case 6-a and equivalent to 5.85 gm. of dry tissue, 1372 mg. of crude hydrochloride were isolated. The once recrystallized product weighed 604 mg. ($[\alpha]_D = +26.6°$); a second

recrystallization gave 477 mg. (Table I). Cohen analysis of an aliquot of the original hydrolysate showed that the total glutamic acid present amounted to 12.0 per cent of the tissue used.

SUMMARY

By the procedures used by Kögl and Erxleben, glutamic acid hydrochloride has been isolated from ten protein samples representing five malignant tumors and one non-malignant tumor. In no case was a fraction obtained the optical rotation of which indicated a $d(-)$-glutamic acid content greater than 4.4 per cent. The glutamic acid isolated amounted to 47 to 70 per cent of the total based on the Cohen analysis for glutamic acid. Our results, therefore, do not support the claim of Kögl and Erxleben with respect to the presence of large amounts of racemized glutamic acid in tumors.

BIBLIOGRAPHY

1. Kögl, F., and Erxleben, H., Z. physiol. Chem., 258, 57 (1939).
2. Kogl, F., Erxleben, H., and Akkerman, A. M., Z. physiol. Chem., 261, 141 (1939).
3. Kögl, F., and Erxleben, H., Z. physiol. Chem., 261, 154 (1939).
4. Kögl, F., Erxleben, H., and Herken, H., Z. physiol. Chem., 263, 107 (1940).
5. White, J., and White, F. R., J. Biol. Chem., 130, 435 (1939).
6. Arnow, L. E., and Opsahl, J. C., Science, 90, 257 (1939).
7. Ottawa, H., Z. Krebsforsch., 49, 677 (1940).
8. Dittmar, C., Z. Krebsforsch., 49, 441 (1939).
9. Konikova, A., Nature, 145, 312 (1940).
10. Chibnall, A. C., Rees, M. W., Tristram, G. R., Williams, E. F., and Boyland, E., Nature, 144, 71 (1939).
11. Graff, S., J. Biol. Chem., 130, 13 (1939).
12. Dittmar, C., Z. Krebsforsch., 49, 397 (1939).
13. Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E., Biochem. J., 34, 285 (1940).
14. Johnson, J. M., J. Biol. Chem., 132, 781 (1940); 134, 459 (1940).
15. Graff, S, Rittenberg, D., and Foster, G. L., J. Biol. Chem., 133, 745 (1940).
16. Behrens, O. K., Lipmann, F., Cohn, M., and Burk, D., Science, 92, 32 (1940).
17. Cohen, P. P., Biochem. J., 33, 551 (1939).
18. Sc. News Letter, 36, 67, 213 (1939).
19. Dohan, J. S., J. Biol. Chem., 135, 793 (1940).

PHOTOMETRIC DETERMINATION OF ESTROGENS

III. A PROCEDURE FOR THE ESTIMATION OF THE ESTROGENS OF PREGNANCY URINE

BY CARL BACHMAN AND DOROTHY SEYMOUR PETTIT

*(From the Department of Obstetrics and Gynecology and the
Gynecean Hospital Institute of Gynecologic Research,
University of Pennsylvania, Philadelphia)*

Various color reactions have been proposed for the estimation of estrogenic hormones. In some of these, the estrogens yield colored solutions which cannot be distinguished from those obtained with other phenols; their application to the determination of estrogens in urine extracts therefore requires a complete separation of the hormones from accompanying phenolic impurities. To our knowledge, the difficulties inherent in such a purification have not been overcome to a sufficient extent to permit reliable hormone determinations.[1] In contrast to these phenol reactions, the color test of Kober (2) and its various modifications (3–8) seem to be quite specific, since no urinary constituent has thus far been shown to yield with their use a pigment having the same absorption characteristics as those obtained with the estrogens. Nevertheless, if the Kober reaction is carried out upon such urinary extracts as have so far been available for quantitative estrogen determinations, the absorption maxima characteristic of the hormones are obscured by the presence of large amounts of colored impurities. The problem of analyzing such absorption spectra for the component parts derived from the estrogens has been attacked in two ways. Kober (5) and Cohen and Marrian (9) treated the crude color products with hydrogen peroxide; this reagent abolishes preferentially, although in our experience by

---

[1] A partial solution of this problem has recently been reported by Talbot *et al.* (1), who have described a procedure for the estimation of a phenolic ketone, probably estrone.

TABLE I

Partition of Estrogens (100 to 300 γ) between Immiscible Solvent Pairs

Solvent pairs		Fraction of total estrogen in (b) after equilibration		
Aqueous phase, 100 cc. (a)	Organic phase, 100 cc. (b)	Estriol	Estradiol	Estrone
		per cent	per cent	per cent
Water	Benzene*	22	100	100
20% NaCl	"	67		
1.0 N NaOH†. . . .	"	0	4	18
0.1 " "	"	0	19	55
0.1 " " in 20% NaCl..	"		82	96
0.05 N NaOH in 20% NaCl .	"		97	96
2.0 N NH₄OH	"	0	71	
1.0 " "	"	0	81	93
0.1 " " .	"	0	81	96
9.0% Na₂CO₃	"	2	98	100
0.9% "	"	2		
0.09% "	"	2		
9.0% NaHCO₃	"	40		
0.9% "	"	17		
0 9% " in 0.9% Na₂CO₃.	"	12		
10 0 N HCl..	"	2	70	91
6.5 N HCl	"	10	98	100
4.0 " "	"	12	98	100
0.5 " " in 20% NaCl	"	67		
4:1 H₂SO₄:H₂O‡	"			0
2:1 " : "	"			60
1:1 " : "	"		77	94
4:5 " : "	"		90	
2:3 " : "	"		90	
1:2 " : "	"		02	98
20% ethanol‡..	"	16		
10% "	"	13		
Water	Ethyl ether	96		
1.0 N NaOH...	" "	0	38	38
0.1 " "	" "	3	67	67
0.1 " " in 20% NaCl...	" "			95
0.02 N NaOH	" "			91
0.01 " "	" "			95
9.0% Na₂CO₃ .	" "	80	98	98
9.0% NaHCO₃	" "	100	100	100

TABLE I—*Concluded*

Solvent pairs		Fraction of total estrogen in (b) after equilibration		
Aqueous phase, 100 cc. (a)	Organic phase, 100 cc. (b)	Es-triol	Es-tradiol	Es-trone
		per cent	per cent	per cent
Water.. . . .	Petroleum ether (b.p. 35–60°)	0	44	77
50% methanol‡	" "		0	6
50% ethanol‡	" "	0	0	7
40% "	" "	0	3	12
30% "	" "	0	8	36
Water	Petroleum ether: benzene (1:1)	0		
30% ethanol‡	" "	5	76	98

* The figures for partition in benzene have been corrected for a slight enhancement (1 to 3 per cent) of the color ordinarily obtained with estrogens in the Kober reaction when tested in the presence of a benzene residue.

† Higher concentrations of NaOH yield no more complete extractions of estrone from benzene than are obtained with a 1.0 N solution.

‡ The proportions represent volumes.

no means exclusively, the absorption derived from the impurities. Venning *et al.* (4) attempted to estimate, and to correct for, the portion of the absorption caused by the contaminants. Their method is based upon observations indicating that the spectra of the contaminants are the same for all types of urine. Our own experience is that these spectra vary too much among different urines to permit reliable determinations of the estrogens if the necessary correction is large. We have therefore attempted to develop a fractionation procedure which would remove most of the chromogenic impurities of urine extracts without reducing their estrogen content.

As a preliminary to such an investigation we determined the distribution coefficients of the estrogens known to occur in human pregnancy urine—estriol, α-estradiol, and estrone—for a number of pairs of immiscible solvents. The data are listed in Table I. Many of the solvents studied have previously been utilized in the

isolation of the estrogens, but quantitative distribution data have thus far been reported in only a few instances (9 12). Among the procedures tested which have not yet been applied to the purification of estrogens was their distribution between strong mineral acids and organic solvents. This was suggested by the work of Wieland and Seibert (13) who utilized the differences in the stabilities of oxonium salts for the separation of a mixture of mono-, di-, and trihydroxy bile acids. While we did not find sufficient differences among the oxonium salts of the estrogens to permit their separation from each other except in the case of estriol, our later studies with urine extracts indicated that many impurities could be separated from estrone and estradiol in this way.

Since it had to be anticipated that distribution coefficients of pure estrogens might be altered significantly by the presence of urinary contaminants, the effect of such impurities upon solubility ratios was studied for those solvents which, according to our data, appeared to be promising for the purification of extracts. On the basis of these studies a procedure was developed which gave good recoveries (about 90 per cent) of estrogen added to various specimens of pregnancy urine. This procedure is outlined in the accompanying diagram (see p. 693).

For the extraction of hydrolyzed urine, ethyl ether or benzene was found to be suitable. With these solvents, estrone and estradiol are quite readily extracted. Since estriol is considerably more soluble in aqueous media than it is in benzene, its complete removal from urine requires either very large volumes of benzene or the use of a continuous extraction apparatus. Saturation of the urine with sodium chloride, however, makes it possible to effect almost complete extraction with the use of only 4 volumes of benzene. Slightly higher yields are obtained if the urine is extracted with ether, but the final products in this case are somewhat more contaminated by chromogenic impurities. Moreover, in the extraction of urine with ether, troublesome emulsions are frequently encountered, whereas benzene and urine that is saturated with salt always separate very readily.

Since the solubilities of estriol differ very much from those of estradiol and estrone, it did not seem feasible to purify the urinary estrogens by distribution procedures without separating estriol

from the other hormones.   This separation is readily effected by extracting the benzene solution of a urinary extract with sodium carbonate.[2]   If water is substituted for the sodium carbonate solution, a much cleaner estriol fraction is obtained, but the removal of estriol is not always complete.   However, if this step is applied to a partially purified estriol fraction, the hormone can be quantitatively extracted from benzene with water.   Estriol

Hydrolyzed urine, diluted and saturated with sodium chloride; extracted with benzene; benzene concentrated

or

Hydrolyzed urine, diluted and extracted with ether; ether evaporated; residue dissolved in benzene

Benzene solution, extracted with sodium carbonate

Sodium carbonate phase, acidified and extracted with ether

Benzene phase, washed with strong sulfuric acid, then extracted with sodium hydroxide

Aqueous phase discarded

Benzene phase discarded

Ether phase, washed with sodium bicarbonate and evaporated; residue dissolved in benzene; benzene washed with bicarbonate and extracted with water

Aqueous phase, acidified and extracted with benzene

Benzene phase discarded

Aqueous phase discarded

Water phase, evaporated; residue, estriol fraction (T)

Benzene phase, concentrated, then washed with sulfuric acid and sodium carbonate; evaporated; residue, estrone-estradiol fraction (OD)

fractions obtained from pregnancy urine in this way have chromogenic properties quite similar to those of the pure hormone.   These fractions have been designated as T fractions in the following sections.

In the purification of the remaining estrogens we have relied

[2] We had already adopted this procedure when Mather (12) reported his independent observation that sodium carbonate will remove estriol quantitatively from a benzene solution of estrogens.

mainly upon their weakly acidic properties and their relatively low solubility in mineral acids. The use of rather strong sulfuric acid removes much pigmented and chromogenic material from the

TABLE II

Recovery of Pure Estrogens

Estrogen added to (volume of substrate, 100 cc.)	Amount of added estrogen (a)	Total estrogen found		Recovery of added estrogen	
		Estriol fraction (T)	Estrone-diol* fraction (OD)	Amount (b)	$\frac{(b)}{(a)}$
	γ	γ	γ	γ	*per cent*
1. Benzene	2000 (T)	1910	0	1910	96
	175 (D)	0	155	155	89
	180 (O)	0	170	170	94
2. Crude benzene ex-tract of hydro-lyzed mid-preg-nancy urine	0		40		
	64 (D)		100	60	94
	61 (O)		97	57	93
3. Hydrolyzed mid-pregnancy urine	0	350	43		
	2000 (T)	2160	43	1810	91
	64 (D)	350	100	57	89
	61 (O)		100	57	93
4. Hydrolyzed late pregnancy urine	0	760	146		
	2000 (T)	2510	143	1750	88
	64 (D)	760	206	63	99
	61 (O)	750	202	59	97
5. Hydrolyzed late pregnancy urine	0	1500	86		
	2000 (T)	3130	82	1630	82
	64 (D)	1500	143	61	95
	61 (O)		141	59	97
6. Hydrolyzed late pregnancy urine	0	4230	154		
	2000 (T)	6050	150	1820	91
	64 (D)	4200	214	64	100
	61 (O)		210	60	98

T = estriol; D = estradiol; O = estrone.

* The estrone-estradiol recoveries have been corrected in the same manner as in Table I.

benzene solutions of urine extracts containing these two hormones. Similar but less thorough purification can be obtained with the use of strong hydrochloric acid. Since pregnancy urine contains very little estradiol (14), we have not attempted to separate this

hormone from the accompanying estrone, but have determined the sum of these estrogens. The final products, designated OD fractions (indicating their content of estro*ne* and estra*diol*), yield pigments in the Kober reaction only slightly less pure than those obtained with the T fractions.

The adequacy of our extraction and purification procedures was tested by a series of recovery experiments. These have been summarized in Table II. It will be seen that our recoveries of estriol added to hydrolyzed urine averaged 88 per cent, those of estradiol 96 per cent, and those of estrone 96 per cent. These

TABLE III

Chromogenic Properties of Urine Extracts. Comparison of Results Obtained with Two Different Reagents

Urine extract	Volume equivalent of original urine	Color product			
		Kober reagent		Phosphoric acid reagent*	
		ϵ_{520}†	Estrogen equivalence	ϵ_{540}‡	Estriol equivalence
	cc.		γ		γ
Estriol fraction, T .	2	0 098	15.0	0 106	15 7
" " " ...	4	0 123	18 8	0.116	17 3
Estrone-diol fraction, OD	50	0.088	13.4	0.020	(0)
" " "	40	0 168	25.6	0 039	(0)

* Specific for estriol.

† ϵ_{520} for 1 γ of estrogen = 0.00654.

‡ ϵ_{540} for 1 γ of estriol = 0.00672; of estrone = 0 00129; of estradiol = 0 00135.

experiments indicated, moreover, that a complete separation of estriol from the other two estrogens was accomplished. Additional evidence for the completeness of this separation was adduced by testing the final extracts with the use of color reactions specific for one or the other of the individual hormones, rather than with the Kober test, which yields the same characteristic color with all three estrogens. The behavior of the final extracts with a reagent specific for estriol (7) has been illustrated in Table III. The data show that estriol is the only hormone present in our T (estriol) fractions, and that it is absent from the OD (estrone-estradiol) fractions. Further studies showed that the estrone-

estradiol fractions, but not the estriol fractions, form a purple
pigment upon treatment with Zimmermann's m-dinitrobenzene
reagent (15); the presence of estrone in the estriol fractions can
therefore be excluded.[3]

The spectral purity of the colored solutions formed by our ex-
tracts in the Kober reaction was determined by photometric
measurements carried out in two regions, at 520 and at 420 mμ.
While the estrogens show maximal absorption at 520 mμ, urinary
impurities absorb more strongly at the shorter wave-lengths.
The ratio $\epsilon_{520} : \epsilon_{420}$ therefore provides a sensitive index of the
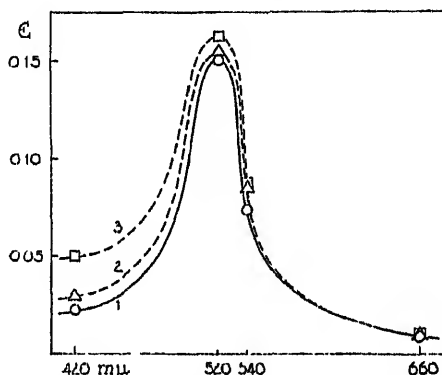
Fig. 1. Absorption characteristics of colored solutions obtained in the
Kober reaction with pure estrogens and with extracts of mid-pregnancy
urine. Equivalent amounts (23 γ) of estrogen are represented in each
curve. Curve 1, pure estrogen, ratio $\epsilon_{520} : \epsilon_{420} = 6.5$; Curve 2, estriol (T)
fraction of urine, ratio $= 5.8$; Curve 3, estrone-estradiol (OD) fraction,
ratio $= 3.2$.

presence of urinary contaminants. With pure estrogen the
ratio is constant only for rather narrow ranges of estrogen con-
centrations, since the extinction at 520 mμ but not at 420 mμ is
proportional to the amount of hormone tested. For estrogen
concentrations that yield ϵ_{520} values between 0.10 and 0.25,
$\epsilon_{520} : \epsilon_{420}$ amounts to about 6.5 under our experimental con-
ditions. The ratios for our T and OD fractions vary between 3.0
and 6.5 (see Fig. 1 and Table IV). Solutions having ratios falling

[3] Because many OD fractions yield colors in the Zimmermann reaction
closely resembling those given by pure estrone, we are currently investi-
gating the feasibility of employing this reaction for estimating the estrone
content of these fractions.

within this range are indistinguishable visually from those obtained with pure hormone. Even with solutions possessing ratios as low as 3.0 the error involved in determining the estrogen content directly from the extinction at 520 mμ probably does not exceed 10 per cent.[4] Below this point, however, the error increases markedly as the value of the ratio falls. Since we do not believe it feasible to correct accurately for this error, our method is limited to urines containing sufficient estrogen in relation to impurities to yield with it Kober colors possessing ϵ_{520} : ϵ_{420} ratios greater

TABLE IV

Chromogenic Properties of Urine Extracts. Purity of Colors Obtained with Kober's Reagent

Urine extract			Color product			Estrogen found	
Maturity of pregnancy	Volume equivalent of original urine	Fraction tested*	ϵ_{520}†	ϵ_{420}	$\dfrac{\epsilon_{520}‡}{\epsilon_{420}}$	Amount	Probable over-estimate
mos.	*cc.*					γ	*per cent*
4	8	T	0 146	0.054	2.7	22.3	13.2
6	8	"	0.183	0.040	4 6	28.0	3.4
9	2	"	0.185	0.030	6.2	28.3	0.9
10	1	"	0.260	0.046	5.7	40.0	1.3
8	30	OD	0.156	0.054	2.9	23.8	11.4
9	20	"	0.205	0.062	3.3	31.3	9.0
10	20	"	0.225	0.050	4.5	34 4	3.7

* T = estriol fraction; OD = estrone-diol fraction.

† ϵ_{520} for 1 γ of estrogen = 0.00654.

‡ ϵ_{520} : ϵ_{420} for estrogen = 6.5.

[4] Venning *et al.* (4) found that the ϵ_{520}:ϵ_{420} ratio of the Kober products obtained with urinary impurities was approximately 0.5, a figure which we can confirm. From this, it may be calculated that the estrogen component of the extinction at 520 mμ for crude Kober products derived from estrogenic extracts equals $1.083(\epsilon_{520} - 0.5\epsilon_{420})$, where ϵ_{520} and ϵ_{420} represent observed values. The presence of contaminants affects only the estimations of *preformed* urinary estrogens. No similar error is involved in estimates of the recoveries of estrogens which have been *added* to urine (see the recovery experiments of Table II). Such estimates are based upon the increase of absorption at 520 mμ exhibited by a color product derived from urine containing added hormone over that of a product derived from a control specimen. The error arising from the presence of small amounts of impurities is the same for each member of the pair; therefore the difference in their optical densities at 520 mμ is a direct measure of their difference in estrogen content.

than 3.0. This condition is fulfilled if the urine contains estriol in concentrations greater than 1000 γ, or estrone (plus estradiol) in amounts exceeding 500 γ per liter.

The above considerations are valid only if it can be shown that the reactions of Kober's solution with estrogens and with urinary impurities proceed independently. Data in support of this are listed in Table V, which shows that the absorption equivalent of a given amount of pure estrogen is the same if measured in the absence or in the presence of a urinary extract.[5] Table VI shows that the extinction at 520 mμ of a urinary extract treated with Kober's reagent is proportional to the amount of extract tested, as is the case with pure estrogens.

TABLE V

Chromogenic Properties of Urine Extracts. Absence of Substances Influencing Development of Color between Pure Estrogen and Kober's Reagent

Urine extract*	Volume equivalent of original urine	Amount of estrogen added to extract	Color product		Amount of added estrogen found
			ϵ_{520}†	Estrogen equivalence	
	cc.	γ		γ	γ
T fraction only	1	0	0.191	29.2	
Same + estriol.. ..	1	41.5	0.471	72.0	42.8
OD fraction only.. .	40	0	0.229	35.0	
Same + estrone	40	14.8	0.328	50.0	15.0

* T = estriol fraction; OD = estrone-diol fraction.

† ϵ_{520} for 1 γ of estrogen = 0.00654.

To obtain further information concerning the purity of our T fractions, the procedure was carried out with urine of late pregnancy on a preparatory scale. Colorimetric assay indicated that more than half of the T fraction consisted of estriol. 57 per cent of this chromogenic material was obtained in crystalline form. The melting point (266-272° corrected) was raised by further recrystallization to 276°.

[5] In the Kober reaction as modified by Venning the intensity of the color developed by estradiol is somewhat diminished if there is an excess of estrone present in the sample being tested. Since such a proportion of estrone to estradiol prevails in pregnancy urine, fractions containing these two hormones may yield less than their actual content of total estrogen if tested by Venning's method. Owing to the small amounts of estradiol involved, however, the error is small (about 3 per cent) in comparison with the total amount of estrogen present in these fractions.

The claimed specificity of the Kober test can be convincingly demonstrated only by a comparison of assay data obtained by colorimetric and biological methods. We have not assayed our final products by both procedures, but have compared in Table

TABLE VI

Chromogenic Properties of Urine Extracts. Proportionality of Extinction at 520 Mμ for Varying Amounts of T (Estriol) Fraction Tested with Kober's Reagent

Volume equivalent of original urine	Color product		
	ϵ_{520}*	Estriol equivalence	
		Total	Per cc.
cc.		γ	γ
5	0.106	16.2	3.2
10	0.218	33.3	3.3
15	0.320	48.9	3.3
20	0.420	64.2	3.2

* ϵ_{520} for 1 γ of estrogen = 0.00654.

TABLE VII

Estrogen Content of Urine of Pregnancy

Maturity of pregnancy	Photometric data, authors' method		Bioassay data of Smith and Smith (14)	
	Estriol	Estrone-diol	Estriol	Estrone-diol
mos.	γ per l.	γ per l.	γ per 24 hrs.	γ per 24 hrs.
4	2,800	270?	2,000	220
6	3,500	270?	4,000	510
7			7,500	565
7	3,450	270?	10,000	330
8	7,600	710	10,000	960
8		580	10,000	330
9	11,600	1500	11,100	742
9	14,100		12,500	270
10	16,500	400	26,500	720
10	40,000	2870	30,000	520

VII colorimetric determinations for different periods of gestation with the bioassay data reported by Smith and Smith (14). The values are of the same order of magnitude.

The colorimetric procedure described in this report is believed to be simpler and more accurate than the biological assay of

urinary estrogens.   However, the range of applicability of the
chemical method in its present form is considerably more limited,
as it cannot be used for estrogen determinations in non-pregnancy
urine.

In this discussion we have not touched upon the errors due to
losses of estrogens taking place during the hydrolysis of urine.
These errors are of unknown magnitude and will affect estrogen
determinations carried out not only by chemical but also by
biological methods.

EXPERIMENTAL

Solvents and Apparatus—Since commercial solvents contain
impurities that interfere with the colorimetric determinations of
estrogens, all solvents used must be purified.

Ether, u.s.p., was shaken with a solution of ferrous sulfate (1
per cent) in water, washed with water, and then distilled.   The
purified solvent was stored in the cold.

Benzene, grade "pure," redistilled.

Petroleum ether, b.p. 35–60°, was treated with concentrated
sulfuric acid for 10 days, separated from and washed free of acid,
and distilled.

All vessels used for distillations and for hydrolysis were equipped
with ground glass joints.   No lubricant was used on the stop-
cocks of the separatory funnels.

Distribution Experiments—The concentrations of estrogen[6] and
the volumes of solvent used have been indicated in Table I.   In
general, the estrogen was first dissolved in a small volume of
ethanol (1 to 3 cc.) and the latter then added to the organic
solvent to be tested.   After equilibration, the organic phase was
separated and taken to dryness.   The residue obtained was taken
up in a measured volume of ethanol; an aliquot of this solution
was then transferred to a colorimeter tube, evaporated to dryness,
and tested with a color reagent (see below).

Hydrolysis of Urine—The pH of the urine was brought to 2
(thymol blue) by the addition of hydrochloric acid.   5 volumes
per cent of concentrated hydrochloric acid were then added, and

---

[6] The melting points of estriol, α-estradiol, and estrone used for this
purpose, for the recovery experiments, and for the preparation of color
standards, have been noted in Paper I (6).

the mixture boiled for 1 hour under a reflux (16).   This technique of hydrolysis of pregnancy urine gave as satisfactory yields of free estrogen as two alternative methods which were tested (17, 18). It was selected principally because the dark hydrolysates obtained with its use appeared to provide a good test of any proposed method of purification.   In the recovery experiments, the urine hydrolysates were seeded with crystalline estrogens that had been dissolved in small volumes of ethanol (not more than 3 cc.).

Extraction of Urine—100 cc. of freshly hydrolyzed urine were cooled and diluted with an equal volume of distilled water.   56 gm. of sodium chloride were added.   The resultant solution was extracted four times with an equal volume of benzene.   The combined benzene extracts were washed with 0.02 volume of a freshly prepared 9 per cent solution of sodium bicarbonate (anhydrous) and concentrated to about 35 cc.

In an alternative procedure the diluted hydrolysate, without the addition of salt, was extracted with 1 equal volume and 2 half volumes of ether.   The combined ether extracts were washed with 0.02 volume of 9 per cent sodium bicarbonate and taken to dryness.   The residue was dissolved in a minimal volume of ethanol (less than 0.5 cc.), and the solution diluted with 35 cc. of benzene.

Fraction T—The benzene extract (35 cc.) was extracted once with 1 and twice with 0.5 volume of 9 per cent sodium carbonate (anhydrous) and once with 0.1 volume of water.   The purification of the benzene phase will be described below.   The aqueous extracts were combined, acidified to a pH of less than 6 with hydrochloric acid, and then extracted three times with 0.5 volume of ethyl ether.   The combined ether extracts were washed twice with 0.1 volume of 9 per cent sodium bicarbonate solution and taken to dryness.   The residue was dissolved in 0.5 cc. or less of ethanol; the solution was diluted with 50 cc. of benzene and washed with 1 cc. of 9 per cent sodium bicarbonate.   The benzene phase was transferred to a clean separatory funnel and extracted with three equal volumes of water.   The aqueous phase was evaporated to dryness *in vacuo*.   The residue was dissolved in a measured volume of ethanol, from which aliquots were transferred to colorimeter tubes and evaporated to dryness for testing.

Fraction OD—The benzene extract (35 cc.), after the removal of

Fraction T, was washed once with 0.25 volume of diluted sulfuric acid (4 parts by volume of the concentrated acid to 5 parts of water) and twice with 0.5 volume of water. It was then extracted four times with an equal volume of N sodium hydroxide. The alkaline extracts were combined, acidified to a pH of 6 or less with hydrochloric acid, and then extracted once with 1.5 and twice with 0.75 volume of benzene. The combined benzene extracts were concentrated to about 50 cc., and were then washed successively with 0.25 volume of diluted sulfuric acid (4:5), twice with 0.5 volume of 9 per cent sodium carbonate solution, and twice with 0.5 volume of water. The benzene was evaporated to dryness and the residue taken up in a measured volume of ethanol, from which aliquots were transferred to colorimeter tubes for evaporation and test.

Color Reagents and Photometry—The T fractions were tested with Kober's phenolsulfonic acid reagent, as modified by Venning *et al.* (4), or with a specific phosphoric acid reagent (7). The OD fractions were usually assayed with the Kober reaction as modified by one of us (6). In the 17-ketosteroid determinations the procedure of Callow *et al.* (19) was followed.

The aliquots of the T and OD fractions used for colorimetric assays contained 10 to 60 γ of estrogens. The photometric measurements were made with an Evelyn photoelectric photometer in the manner described in Paper I (6).

The final color products obtained by testing the OD fractions with the Kober reagent were further purified by washing with 0.5 volume of benzene. (After separation of the benzene, the colored solutions may be found clouded by the presence of a small residuum of emulsified benzene; they can be clarified under reduced pressure.) This procedure removes impurities without affecting the pink pigment produced by the estrogens, provided, however, that the value of the ratio ϵ_{520} : ϵ_{420} of the color product is not lower than 2.0 before washing. If the OD fraction contains a higher concentration of impurity (ϵ_{520} : ϵ_{420} less than 2.0), an appreciable amount of estrogen color is also removed.

SUMMARY

A method is described whereby urinary estrogens may be extracted quantitatively in a form pure enough to yield, with

appropriate color reagents, products closely resembling those obtained with pure hormones. In the procedure, estriol is separated from estrone and estradiol. The method can be used for determinations of estriol when this substance is present in the urine in concentrations exceeding 1000 γ per liter, and for estimating the sum of estrone and estradiol when this exceeds 500 γ per liter; *i.e.*, pregnancy urine from about the 4th month of gestation to term. Aside from losses of hormone occurring during the hydrolysis of urine, which are of unknown magnitude and affect all methods of determination, the estimations of urinary estrogens obtained with our procedure are accurate within limits of about ±10 per cent. The method is simple enough for use in clinical investigations of the urinary excretion of estrogens.

In the development of the method, data have been obtained concerning the distribution coefficients of estrone, α-estradiol, and estriol for a large number of pairs of immiscible solvents.

BIBLIOGRAPHY

1. Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.*, **134**, 319 (1940).
2. Kober, S., *Biochem. Z.*, **239**, 209 (1931).
3. Cartland, G. F., Meyer, R. K., Miller, L. C., and Rutz, M. H., *J. Biol. Chem.*, **109**, 213 (1935).
4. Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, J. S. L., *J. Biol. Chem.*, **120**, 225 (1937).
5. Kober, S., *Biochem. J.*, **32**, 357 (1938).
6. Bachman, C., *J. Biol. Chem.*, **131**, 455 (1939).
7. Bachman, C., *J. Biol. Chem.*, **131**, 463 (1939).
8. Szego, C. M., and Samuels, L. T., *Proc. Soc. Exp. Biol. and Med.*, **43**, 263 (1940).
9. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, **28**, 1603 (1934).
10. Butenandt, A., *Z. physiol. Chem.*, **191**, 127 (1930).
11. Westerfeld, W. W., Thayer, S. A., MacCorquodale, D. W., and Doisy, E. A., *J. Biol. Chem.*, **126**, 181 (1938).
12. Mather, A., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133**, p. lxiii (1940).

13. Wieland, H., and Seibert, W., *Z. physiol. Chem.*, **262**, 1 (1939).
14. Smith, G. V., and Smith, O. W., *Am. J. Obst. and Gynec.*, **39**, 405 (1940).
15. Zimmermann, W., *Z. physiol. Chem.*, **233**, 257 (1935).
16. Cherry, T. H., and Bernstein, M. J., *Proc. Soc. Exp. Biol. and Med.*, **40**, 688 (1939).
17. Smith, O. W., Smith, G. V., and Schiller, S., *Endocrinology*, **25**, 509 (1939).
18. Callow, N. H., Callow, R. K., Emmens, C. W., and Stroud, S. W., *J. Endocrinology*, **1**, 76 (1939).
19. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, **32**, 1312 (1938).

THE METABOLISM OF SULFUR

XXVII. THE DISTRIBUTION OF SULFUR IN THE ULTRA-FILTRATES OF BLOOD PLASMA*

BY BARKER H. BROWN AND HOWARD B. LEWIS

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

The distribution of the non-protein sulfur of the blood, as recorded in the literature, varies greatly. While, to some extent, these variations may be related to species differences, they are undoubtedly due also to lack of uniformity in the analytical procedures employed. Most of the more recent studies have been concerned with the inorganic sulfate content of blood[1] (1, 2). The presence of organic sulfur in blood as a constituent of the molecule of glutathione has been recognized, but despite the very considerable volume of data, the values are difficult to interpret because of lack of specificity of the methods commonly employed for its determination. The estimation of ergothioneine, which would also contribute to the organic sulfur fraction, has not been satisfactory.

In experiments with animals to which sulfur-containing compounds had been administered, we were interested in the partition of the non-protein sulfur of the blood. Such partitions of urinary sulfur have been determined commonly, but to our knowledge, this procedure, as applied to blood, has not been used in the study of the intermediary metabolism of the sulfur compounds.

It was also hoped that the demonstration of the presence of cystine in blood and its quantitative determination might be

[1] The papers of Power and Wakefield (1, 2) afford excellent critical reviews of the methods for the determination of sulfate sulfur in blood and data concerning the sulfate sulfur of human blood.

accomplished, a demonstration previously unconvincing (3, 4). Since cystine, if it is included among the amino acids normally present in blood, would be present in exceedingly small amounts, it was evident that loss of cystine associated with the usual deproteinization of blood prior to the determination of the non-protein sulfur (5, 6) must be prevented. It was also desirable to avoid dilution of the blood which is usually necessary in deproteinization procedures.

Consideration of these facts convinced us that ultrafiltration might be well adapted to our purposes. In ultrafiltration, loss of sulfur compounds by adsorption on the precipitated protein is avoided, dilution is unnecessary, and the possibility of secondary changes in the sulfur compounds by the action of deproteinizing reagents is minimized. In the present paper are reported studies of the partition of the non-protein sulfur of ultrafiltrates of blood plasma of various species. It has also been possible to determine cystine (*i.e.*, the chromogenic factor in the Sullivan reaction with naphthoquinonesulfonic acid) in these filtrates.

The ultrafiltration procedure of Greenberg and Gunther (7) was selected as simple, rapid, and efficient. The chief modifications were the use of larger collodion (parlodion, Mallinckrodt) sacs of 25 cc. capacity for ultrafiltration membranes and of larger receiving tubes (70 cc. capacity) in order to increase the usefulness of each filter. It was possible to obtain an ultrafiltrate corresponding in volume to from 40 to 60 per cent of the plasma in 3 to 5 hours. All ultrafiltrates were tested for the presence of substances precipitable by trichloroacetic acid with negative results. Shortly after we had standardized our ultrafiltration procedure, Gaebler (8) described an ultrafilter by which 67 per cent of the serum could be filtered in 2 hours. If this could be adapted to the samples of blood obtainable in metabolism experiments with small animals, its use would be preferable because of its greater rapidity and effectiveness.

For the sulfur partition studies, plasma was obtained by centrifugation of oxalated plasma. Since the amount of cystine in blood was found to be very small and since it was planned to remove and analyze samples of blood from the same animal repeatedly, it was necessary to employ as small a sample of blood for each analysis as was possible and to obtain a maximal volume

of plasma for ultrafiltration, when cystine was to be determined. In preliminary experiments, it was observed that the corpuscular mass remaining after centrifugation was more compact and that a larger volume of plasma was secured when fluoride was used as an anticoagulant. Thereafter in order to obtain maximal volumes of plasma (and ultrafiltrates), we employed a mixture of fluoride and oxalate in the preparation of plasmas, in ultrafiltrates of which we desired to determine cystine. For each 10 cc. of blood, 400 mg. of sodium fluoride and 0.2 to 0.3 cc. of a 20 per cent solution of potassium oxalate were placed in the centrifuge tube in which the blood was collected and centrifuged. This fluoride-oxalate plasma was not satisfactory for the determination of the sulfur partition in the ultrafiltrates, since the presence of the fluoride appeared to interfere with the precipitation of the benzidine sulfate. It was, however, employed successfully in the determination of cystine.

The choice of a method for the determination of sulfur in the ultrafiltrate was not easy. A colorimetric reaction suitable for the determination of small amounts of sulfur and adapted to photometric methods seemed essential. After many trials, the benzidine precipitation method was selected. The benzidine content of the precipitate was determined essentially by the method of Cuthbertson and Tompsett (9) as modified by Pirie (10), which involves diazotization of the benzidine followed by coupling with thymol in alkaline solution. The percentage transmission of light through the red solution was determined in a Zeiss Pulfrich photometer (11) used in the vertical position. A 10 mm. cell and a No. S-50 filter were found most satisfactory. The photometric procedure was standardized by measuring the light transmission when known concentrations of benzidine (1 to 10 γ) were similarly diazotized and coupled with thymol.

The use of oxalated plasma is, in general, not entirely satisfactory when benzidine is to be used as a precipitant for the sulfate ion, as in the determination of inorganic and total sulfate sulfur. We were, however, faced with the problem of securing a fluid free from cells as rapidly as possible, since we wished to avoid hemolysis and possible liberation of substances which might yield compounds containing sulfur which would be ultrafiltrable. The use of oxalate and immediate centrifugation seemed the best solution. A part

of the added oxalate is precipitated and it is possible that part may not be ultrafiltrable. The amount of oxalate added was always the same (approximately 25 mg. of potassium oxalate per 10 cc. of blood) and any error should be a constant one. The fact that the results obtained were comparable to those for serum and plasma reported by others leads us to believe that no serious error is introduced by the use of oxalate in our plasma ultrafiltrate analysis. The procedure was designed for use in studies of the metabolism of sulfur-containing compounds and we consider that the values obtained are comparable and capable of interpretation.

Inorganic Sulfate Sulfur—0.5 cc. of an ultrafiltrate of human plasma was added to an equal volume of 20 per cent trichloroacetic acid,[2] 0.1 cc. of sulfate-free water and 2.5 cc. of a freshly prepared 1 per cent solution of benzidine in redistilled acetone were added, and the solution was thoroughly mixed by vigorous stirring. With animal plasmas of a higher sulfate content smaller volumes of ultrafiltrate were employed and water added to give a volume of 0.5 cc. We have preferred to carry out the precipitation in capillary centrifuge tubes of approximately 15 cc. capacity. These tubes, which may be made readily from Pyrex tubing, should have a capillary not over 1 mm. in diameter and from 5 to 10 mm. in length. After thorough mixing, the tubes were allowed to stand in the refrigerator for 0.5 to 2 hours, the time being dependent on the amount of sulfate present. The tubes were then centrifuged at approximately 3500 r.p.m. for 15 minutes.[3] After decantation of the supernatant liquid, the precipitate was washed thoroughly in the tube, first with 85 per cent acetone and subsequently with absolute acetone. It was desirable to use thin stirring rods of wood, since glass or wire rods were observed to scratch the sides of the tube and centrifugation was unsatisfactory. The further treatment of the precipitate was as described by Pirie (10) with the use of the photometer to determine the light transmission of the colored solution. It was, of course, necessary to carry out blank determinations to ascertain the sulfur content of all the reagents used in this and the procedures to be outlined subsequently.

---

[2] The conditions for the precipitation of the benzidine sulfate are essentially those of Hubbard (12).

[3] Procedures for filtration were also developed and used, but in our opinion, centrifugation is more satisfactory.

Total Sulfate Sulfur—1 cc. of human plasma ultrafiltrate (0.3 to 0.5 cc. of plasma ultrafiltrates of other species) was placed in a small porcelain crucible (No. 0 is convenient) with 1 cc. of 10 per cent hydrochloric acid and heated for 1 hour on the steam bath. To the dry material 1 cc. of water was added and the heating was repeated for approximately 1 hour. The contents of the crucible were then dissolved in 3 cc. of 10 per cent trichloroacetic acid with the aid of vigorous stirring. The solution was transferred to a centrifuge tube and any charred material or silica was centrifuged out. An aliquot of the supernatant fluid (usually 1 cc.) was analyzed as already described. *Conjugated sulfate sulfur* was calculated as the difference between the total sulfate and inorganic sulfate sulfur.

Total Sulfur—Aliquots of the ultrafiltrates (usually 0.3 to 0.8 cc. for animal plasma ultrafiltrates and 1 cc. for human) were measured into small porcelain crucibles, 1 cc. of a diluted Benedict's sulfur reagent (15 cc. diluted with sulfate-free water to 50 cc.) was added, the solutions were carefully evaporated to dryness on a steam bath, and the residues were ignited with the precautions usual in macro sulfur determinations. The ignited material was dissolved in 1 cc. of hydrochloric acid (10 per cent) and the solution was evaporated to dryness on the steam bath. Subsequent procedures were the same as those described for the total sulfate sulfur determination.

Experiments with known solutions of potassium sulfate and cystine by the methods outlined demonstrated that with amounts of sulfur comparable to those present in ultrafiltrates satisfactory results could be obtained. In similar experiments in which known amounts of cystine and inorganic sulfate were added to plasma before ultrafiltration, the recoveries were slightly high. The results of a typical experiment are presented in Table I.

A summary of results obtained is presented in Table II, together with values for the partition of sulfate sulfur of normal human sera obtained by Power and Wakefield (1). (This paper should be consulted for a summary and evaluation of the more important determinations of the inorganic sulfate sulfur of human blood sera.) The concentrations of total sulfate and inorganic sulfate sulfur of human plasma ultrafiltrates are comparable to, although slightly lower than, those of human sera reported by these investigators, while the values for conjugated sulfate sulfur are

slightly higher. It is realized that the latter are calculated by difference and the results are therefore less accurate. We believe, nevertheless, that the differences between the total sulfate and inorganic sulfate fractions, although slight, indicate the presence of small amounts of conjugated sulfate in plasma. In a preliminary report (13), available after the completion of the experimental work here reported, Letonoff and Reinhold have presented a study of the partition of the non-protein sulfur of human sera which is summarized in Table II. Deproteinization was effected by uranium acetate. Filtrates obtained by deproteinization with tri-

TABLE I

Recovery of Inorganic Sulfate and Organic Sulfur Added to Plasma

A sample of human plasma was subjected to ultrafiltration and the ultrafiltrate was analyzed. The procedure was repeated with a sample of the same plasma to which potassium sulfate and cystine, each equivalent to 0.83 mg. per cent of sulfur, were added. All values are the averages of closely agreeing duplicate determinations and are reported as mg. per 100 cc. of filtrate.

	Sulfur				
		Sulfate			
	Total	Total	Inorganic	Conjugated	Organic
Plasma ultrafiltrate, no added sulfur compounds (a)	2.78	1 15	0.97	0.18	1.61
Plasma ultrafiltrate, cystine and sulfate added to plasma (b)	4.47	2.01	1.86	0.15	2.46
Difference, (b) − (a).......	1.69	0.86	0.89	−0.03	0.85
Sulfur added........	1.66	0.83	0.83	0	0.83

chloroacetic acid, the agent employed by Power and Wakefield, gave significantly higher values for total, inorganic sulfate, and organic sulfur, while the values for conjugated sulfate sulfur were lower. They also state that the analyses of plasma were comparable to those of sera. As shown in Table II, the total non-protein sulfur content of human sera, as determined in their studies, is very similar to that of ultrafiltrates of human plasma.

The oxidized (sulfate) sulfur of the ultrafiltrates of the other species studied, notably the rabbit, is significantly higher than that of human plasma ultrafiltrates. The organic sulfur fraction

TABLE II

Sulfur Partitions of Plasma Ultrafiltrates of Normal Mammals

All values are expressed as mg. per cent of the plasma ultrafiltrates. The figures in parentheses represent maximum and minimum values. The last two horizontal rows of values represent recent analyses of human sera which are presented for comparison.

Source of blood	No. of animals	No. of samples	Total	Sulfur — Sulfate Total	Sulfur — Sulfate Inorganic	Sulfur — Conjugated	Sulfur — Organic
Rat	28	6	3.85 (3.7-4.1)	2.32 (2.1-2.6)	2.17 (1.9-2.4)	0.20 (0.07-0.4)	1.52 (1.3-1.7)
Hog*	?	1	3.88	2.08	1.89	0.19	1.80
Beef*	?	1	5.64	4.44	3.44	1.00	1.20
Rabbit†	8	30	7.05 (6.0-8.3)	5.36 (4.0-6.9)	4.98 (3.6-6.1)	0.42 (0.1-1.1)	1.65 (1.0-2.1)
Man†	3	6	2.68 (2.47-2.90)	1.07	0.86	0.22	1.60 (1.37-1.75)
" ‡	8	12	2.97 (2.48-3.60)	1.18 (0.93-1.15)	0.99 (0.82-0.97)	0.19 (0.09-0.32)	1.79
Human serum§ (1)	16	16		1.25 (0.97-1.58)	1.17 (0.94-1.49)	0.076 (0.00-0.19)	(1.42-2.57)
" " ‖ (13)	9		2.84 (2.40-3.10)	1.09 (1.00-1.22)		0.80 (0.40-1.19)	0.94 (0.57-1.48)

* Slaughter-house blood.
† Fasting blood.
‡ Obtained after a light breakfast.
§ Trichloroacetic acid filtrate.
‖ Uranium acetate filtrate

does not show any variation characteristic of the species. The inorganic sulfate sulfur values of ultrafiltrates of rabbit and beef plasma are similar to those reported for serum ultrafiltrates of the same species by Meyer-Bisch and Heubner (14), who determined the sulfur microgravimetrically as barium sulfate.

Cystine Content of Plasma Ultrafiltrates—For the determination of the cystine content of ultrafiltrates of fluoride-oxalate plasma, the reaction with naphthoquinonesulfonic acid was utilized. Since, with the small amounts of cystine present, the color developed was slight, we have employed the Zeiss Pulfrich photometer rather than the colorimeter. This instrument has been used by Bushill, Lampitt, and Baker (15) in similar colorimetric determinations with satisfactory results.

After many trials, the following procedure was developed which in our hands has given satisfactory and reproducible results. Since the naphthoquinonesulfonic acid reaction is a difficult one at best, it is necessary to standardize carefully all the procedures. To 6 cc. of ultrafiltrate (if smaller volumes are used, these should be diluted with water to give a volume of 6 cc.), 2 cc. of 10 per cent sodium cyanide (aqueous solution) are added and, after mixing, the solutions are allowed to stand for 10 minutes at 20–25°. During this interval, the sodium sulfite solution (10 per cent in 0.5 N sodium hydroxide) and the quinone solution (0.5 per cent aqueous solution of sodium β-naphthoquinonesulfonic acid) are prepared. At the end of the 10 minute period, add 1 cc. of the quinone solution and *exactly* 10 seconds later 5 cc. of the alkaline sulfite solution with thorough but not too vigorous mixing after the addition of each reagent. The tubes are stoppered and placed in the dark. After color development has proceeded for 30 minutes, 1 cc. of freshly prepared alkaline sodium hydrosulfite (1 per cent in 0.5 N sodium hydroxide) is added and the solution is carefully mixed. After 10 to 15 minutes, the solutions are placed in a 10 mm. cell of the Zeiss Pulfrich photometer with the use of a No. S-53 filter[4] and readings are taken. The standard curve of the photometer was obtained with known amounts of cystine treated as described above.

[4] Bushill and coworkers (15) have shown that the best proportionality between extinction coefficients and cystine content is obtained with Filter S-53. Krijgsman and Bouman (3) advise the use of Filter S-57.

Control experiments with varying amounts of pure cystine and with "synthetic filtrates," which contained sodium chloride, urea, uric acid, glucose, creatinine, and creatine and a mixture of amino acids (including methionine) in amounts comparable to or somewhat greater than those present in plasma, to which cystine was added showed that cystine could be determined with an accuracy of at least 10 per cent and usually within 5 per cent. While this may not be as accurate as is desirable, it must be remembered that the quantities of cystine in blood are exceedingly small. Cystine was added to whole blood in varying amounts, the plasma samples before and after the addition of cystine were subjected to ultra-

TABLE III

Cystine Content of Plasma Ultrafiltrates

All values are expressed as mg. per 100 cc. of the ultrafiltrate of oxalate-fluoride plasma

Type of blood	No. of specimens analyzed	Cystine	Remarks
Hog	3	0.64–0.87	Mixed blood from slaughter-house
Rat	3	0.75–0 86	Heart " " 3 groups of 4–5 rats after 24 hr. fast
Human	6	0.71–1.06	Fasting, no breakfast
"	2	0.89–1 14	Light breakfast
"	3	0.91–1.06	Heavy "
Rabbit*	6	0 93–1.11	24 hr. fast

* Average value, 1.02 mg. per cent.

filtration, and the cystine content of the ultrafiltrates was determined. In a typical experiment, to a sample of hog blood whose plasma ultrafiltrate was found to contain 0.83 mg. per cent of cystine, cystine equivalent to 0.47, 0.94, 1.32, 2.35, 4.71, 9.41, and 14.10 mg. per 100 cc. was added. The extra cystine contents of the plasma ultrafiltrates as determined were 0.69, 0.97, 1.34, 2.77, 5.27, 8.08, and 14.97 mg. per cent respectively. It was felt from the results of these and other similar determinations that the use of this procedure in comparative studies in which sulfur-containing compounds were administered to experimental animals was warranted.

From Table III, it is evident that cystine (*i.e.*, the chromogenic

factor in the naphthoquinonesulfonic acid reaction) is present in very small but detectable amounts in normal plasma ultrafiltrates. The amount was relatively constant in fasting blood and no species differences were observed. A content of cystine of 1.0 mg. per cent (16) would be equivalent to approximately 0.12 mg. per cent of cystine nitrogen. Determinations of the amino nitrogen of our plasma ultrafiltrates by Folin's colorimetric procedure have shown a content of 4 to 12 mg. per cent, with the greater number of the values included within the range of 4 to 7 mg. The average cystine nitrogen of the ultrafiltrates is thus approximately 2 per cent of the total free amino nitrogen, a value not inconsistent with the amount to be expected, if the amino nitrogen of the blood is a mixture of the various amino acids in the proportions present in the more common types of food or body proteins.

Okuda (17) reported a content of 1 mg. per cent of cystine in cow serum as calculated from the —SS— content determined iodometrically. Hess (6) was unable to detect the presence of cystine in human blood. Harding and Cary (5) found little, if any, free cystine in blood plasma of cows but were unable to recover cystine added to plasma satisfactorily. More recently Krijgsman and Bouman (3), using tungstic acid filtrates and the Sullivan method for cystine[5] applied photometrically, found traces only of total cystine in the blood of rats fasted 12 to 72 hours and surprisingly large amounts in the blood of laboratory animals 1 to 4 hours after feeding (up to 14 mg. per cent). After the completion of the work here reported, Fujita and Numata (4) determined cystine, total and preformed, in metaphosphoric acid filtrates of blood and tissues by a photometric application of the reaction of cysteine with dimethylphenylenediamine in the presence of ferric salts. A content of "total cysteine" of 0.8 mg. per cent was found in a single sample of rabbit blood. It is pointed out, however, that the determinations were subject to great error and that part of the cysteine may have originated from postmortal hydrolysis of glutathione. All of the total cysteine was believed to be present as preformed cysteine rather than as cystine. More recently (18)

[5] Sodium thiosulfate is stated to have been used in the final stage of the reaction rather than sodium hydrosulfite as recommended by Sullivan This presumably is a typographic error, since some confusion as to the nomenclature of sodium hydrosulfite exists.

the total cystine content of horse plasma has been reported to be 1.6 mg. per cent, a value significantly higher than that of horse erythrocytes, 0.6 mg. per cent. Hunter and Eagles (19) questioned the presence of free cysteine in blood.

It should be noted that we have made no attempt to determine the possible presence of cysteine in any of the ultrafiltrates. The values reported here and in Paper XXVIII represent "total" cystine (*i.e.*, values obtained by the application of the naphthoquinonesulfonic acid procedure to the ultrafiltrates after reduction with cyanide). Since glutathione is present in the erythrocytes rather than in plasma, it is believed that the possible origin of cystine (or cysteine) from this source is eliminated by the use of ultrafiltrates of plasma. The determination has been found of value in metabolism experiments, as will be detailed in Paper XXVIII.

SUMMARY

1. Ultrafiltrates of plasma have been shown to be well adapted to the study of the non-protein sulfur of blood plasma. The partition of the non-protein sulfur of plasma ultrafiltrates of a number of species is discussed. The values for sulfate sulfur are similar to those obtained with sera by other workers.

2. It has been possible to determine cystine quantitatively in ultrafiltrates of plasma by a photometric adaptation of the color reaction with naphthoquinonesulfonic acid (Sullivan). The total cystine content of the ultrafiltrates of plasma of various species ranged from 0.64 to 1.15 mg. per cent.

BIBLIOGRAPHY

1. Power, M. H., and Wakefield, E. G., *J. Biol. Chem.*, **123**, 665 (1938).
2. Wakefield, E. G., Power, M. H., and Keith, N. M., *Arch. Int. Med*, **63**, 679 (1939).
3. Krijgsman, B. J., and Bouman, E. F., *Arch. néerl. physiol.*, **18**, 458 (1933).
4. Fujita, A., and Numata, I., *Biochem. Z.*, **300**, 264 (1939).
5. Harding, T. S., and Cary, C. A., *Proc. Soc. Exp. Biol. and Med.*, **23**, 319 (1925-26); *Proc Am Soc. Biol. Chem*, *J Biol Chem*, **78**, p. xlix (1928).
6. Hess, W. C, *J. Washington Acad. Sc.*, **19**, 419 (1929).
7. Greenberg, D. M., and Gunther, L., *J. Biol. Chem*, **85**, 491 (1929-30).
8. Gaebler, O. H., *J. Biol. Chem.*, **117**, 397 (1937)

9. Cuthbertson, D. P., and Tompsett, S. L., *Biochem. J.*, **25**, 1237 (1931).
10. Pirie, N. W., *Biochem. J.*, **28**, 305, 1063 (1934).
11. Krebs, W., Clinical colorimetry with the Pulfrich photometer, Jena (1936).
12. Hubbard, R. S., *J. Biol. Chem*, **88**, 663 (1930).
13. Letonoff, T. V., and Reinhold, J. G , *Proc. Am Soc. Biol. Chem.*, *J. Biol. Chem* , **133**, p. lix (1940).
14. Meyer-Bisch, R., and Heubner, W , *Biochem Z* , **176**, 184 (1926).
15. Bushill, J H , Lampitt, L. H., and Baker, L C., *Biochem. J.*, **28**, 1293 (1934).
16. Brown, B H., and Lewis, H. B., *Proc. Soc Exp. Biol. and Med.*, **36**, 487 (1937).
17. Okuda, Y., *Proc Imp Acad. Japan*, **3**, 287 (1927), *J. Dept. Agric. Kyushu Imp. Univ* , **2**, 133 (1929).
18. Numata, I., *Biochem. Z.*, **304**, 404 (1940).
19. Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, **72**, 167 (1927).

THE METABOLISM OF SULFUR

XXVIII. THE CYSTINE CONTENT AND SULFUR DISTRIBUTION OF ULTRAFILTRATES OF PLASMA AFTER THE ADMINISTRATION OF l-CYSTINE AND dl-METHIONINE TO RABBITS *

By BARKER H. BROWN and HOWARD B. LEWIS

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

The course of the metabolism of the naturally occurring sulfur-containing amino acids, cystine and methionine, has been studied frequently by the determination of the changes in the partition of the urinary non-protein sulfur after the administration of the amino acids or their derivatives (2–5). Similar investigations of the changes in the distribution of the non-protein sulfur of blood or plasma have not been made to our knowledge. In Paper XXVII of this series (6), we have shown that the partition of the non-protein sulfur of ultrafiltrates of plasma is carried out readily and that it is possible to determine the small amounts of cystine present in these ultrafiltrates satisfactorily. The present paper is concerned primarily with a study of the changes in the non-protein sulfur of plasma ultrafiltrates after the administration of l-cystine and dl-methionine to rabbits. In the young white rat, growth occurs in the absence of dietary cystine when methionine is available (7). From this and other evidence, it has been assumed that methionine may be converted to cystine in the animal organism (8–11). It was therefore considered to be of particular interest to determine whether the cystine content of plasma ultrafiltrates was changed after the administration of methionine.

717

Young male rabbits of 2 to 3 kilos of body weight were maintained on a constant mixed diet which permitted maintenance of weight or even growth in many cases. The animals were subjected to short preliminary fasts (usually of 24 hours duration) prior to and during each experiment. Blood was obtained by cardiac puncture. Since a considerable amount of blood (30 to 40 cc.) was required for each complete analysis, it was not advisable to withdraw serial samples at frequent intervals on any one experimental day. It was possible to withdraw the amount of blood required for a complete analysis at weekly intervals without injury to the animals. Accordingly one experiment was conducted each week with each animal, in which a single sample of blood was withdrawn at the desired interval after administration of the compound under investigation (*i.e.*, 1, 3, 6 hours, etc.). The constancy of the control values for fasting blood of each animal (Table I) would appear to justify this procedure. For example, in the case of Rabbit 5, thirteen samples of blood taken at weekly intervals were analyzed. The preparation of the plasma, the ultrafiltration procedure, and the methods of analysis have been described in Paper XXVII (6).

The purity of the amino acids administered (*l*-cystine from hair and synthetic *dl*-methionine) was established by analyses. The sodium salts were administered in amounts equivalent in their sulfur content (0.1 gm. of sulfur per kilo of body weight), either orally by stomach tube or by subcutaneous injection. As a further control of the experimental procedure, a simple naturally occurring amino acid which contained no sulfur, glycine, was fed in amounts molecularly equivalent to those of the sulfur-containing amino acids.[1] This served to determine whether flooding the system with amino acids regardless of their sulfur content would influence the partition of non-protein sulfur of the plasma. As is seen by inspection of Table I, the values obtained after glycine was fed were similar in every respect to the fasting control values. The changes in sulfur distribution observed after the administration of cystine or methionine would appear, therefore, to be related directly to the sulfur content of the amino acids fed or injected.

[1] In calculating the glycine equivalent of cystine, 2 moles of glycine were considered equivalent to 1 mole of cystine (*i.e.*, 2 moles of cysteine).

TABLE I

Sulfur Distribution in Ultrafiltrates of Plasma of Rabbits after Administration of dl-Methionine, l-Cystine, and Glycine

Unless otherwise indicated, the animals were fasted 24 hours prior to the experiment. The sulfur-containing amino acids were administered in amounts equivalent to 0.1 gm. of sulfur per kilo and glycine in amounts equivalent in nitrogen content to that of sulfur-containing amino acids. All results are reported as mg. per 100 cc. of ultrafiltrate.

Animal No	Weight*	Sulfur					Notes
		Total	Sulfate			Organic	
			Total	Inorganic	Conjugated		
	kg.						
2	2 2	5.70	4 75	3 68	1.07	0.95	12 hr. fasting control
	2 7	7 12	4 95	4.38	0.57	2 17	Fasting control
	2.3	7 30	5 98	5.45	0 53	1.32	" "
	2 4	7.40	5.89	5.32	0 57	1.51	" "
	2 4	7.81	5.94	5 35	0 59	1 87	3 hrs. after oral glycine
	2 4	10 49	6 27	6.00	0 27	4.22	1 hr. " " methionine
	2 5	13 98	6 23	5 96	0.27	7.75	3 hrs. " " "
	2.6	14 80	7.41	7.20	0.21	7 39	3 " " " "
	2 5	10.95	7 40	6.90	0.50	3.55	6 " " " "
	2 5	11 75	7.40	6 97	0.43	4 35	6 " " " "
3	3 4	6 02	4 70	4 05	0 65	1.32	12 hr. fasting control
	3.3	6.63	4.87	4.74	0.13	1.77	15 " " "
	3 2	6 06	4.37	4.00	0.37	1 69	Fasting control
	3 6	6 61	4 69	4 43	0 26	1.92	48 hr. fasting control
	3.4	7 04	5.28	4.47	0.81	1 76	0 5 hr after oral cystine
	3.3	11.00	6.77	5.52	1 25	4 23	1 hr. after oral cystine
	3 3	8 35	6 56	6.49	0 07	1.79	3 5 hrs. after oral cystine
	3 3	10 59	8 94	8.17	0.77	1.63	6 hrs. after oral cystine
	3.5	7.40	5.65	5.10	0.55	1.75	12 hrs. after oral cystine
5	2.5	7 70	5 94	5.30	0 64	1.76	12 hr. fasting control
	2 8	7.10	5.44	5 27	0 17	1.66	Fasting control
	3 3†	7 43	5.67	5.35	0 32	1.76	" "
	3.6	7.40	5.60	5.25	0.35	1.80	4 hrs. after oral glycine
	3.2	10.48	6 66	6.30	0.36	3.82	1 hr. " " cystine
	3 4	10.70	7.55	7.05	0 50	3.15	3 hrs. " " "
	3 7	9.62	7.32	6 98	0.34	2.30	6 " " " "
	3.0	11.67	6 18	5.60	0.58	5 49	1 hr. " " methionine
	3 2	11.36	6 99	6.64	0.35	4.37	3 hrs. " " "
	2 6	10 30	6 95	6 85	0 10	3.35	6 " " " "
	2 8	13 80	9.35	8 05	0.70	4 45	6 " " " "

* As the experiments were done at weekly intervals, the weight of the animal changed as the experiment progressed.

† Sulfur partition of a trichloroacetic acid filtrate of plasma.

Ten rabbits were used in the study in which twenty-eight samples of blood of fasting control animals, seven after oral glycine, twenty-three after oral cystine, twenty-one after oral methionine, and three and four after subcutaneous cystine and methionine, respectively, were analyzed. In the later experiments, which included most of the parenteral administrations of the amino acids, the cystine content only of the ultrafiltrates was determined. In the consideration of the data presented in Tables I and III, it should be noted that, although similar experiments are grouped in the tables for easy comparison, the chronological order of the various experimental procedures was varied in order to be certain that the metabolic picture had not been altered in the course of the series. At least one analysis on a fasting control was made at the beginning and again at the end of the series of experiments with each animal.

Details of typical experiments with three of the rabbits are presented in Table I. The fasting control values were surprisingly constant with the possible exception of those of conjugated sulfate sulfur. As previously stated (6), we regard these values, obtained by difference, as less accurate than the values for inorganic sulfate or total sulfate sulfur. However, there is no evidence from our experiments that the conjugated sulfate sulfur of plasma ultrafiltrates is significantly altered by the administration of the sulfur-containing amino acids. In Table II, the average changes in the total sulfate and organic sulfur of the entire series of experiments are presented in relation to the control fasting values; i.e., as the percentage of the fasting values of each individual animal. While averages of relatively small series are perhaps not entirely significant, the values in Table II serve to lend emphasis to those of Table I in that they also show a rapid increase in the sulfur content of the plasma ultrafiltrates after either of the sulfur-containing amino acids is fed.

The increase in the oxidized (total sulfate) sulfur is clearly demonstrated 1 hour after cystine is fed, while the organic sulfur was usually at a higher level 1 hour after cystine than after 3 hours. The total sulfate sulfur continued to increase up to 6 hours (and possibly 9 hours, one experiment only), and was somewhat above the fasting level after 12 hours. The organic sulfur, on the other hand, reached its high level early and declined thereafter. This

indicates a rapid oxidation of the sulfur of cystine to sulfate, associated perhaps with a rapid removal of cystine from the blood.

After methionine was fed, the rate of increase of the oxidized sulfur was less marked than after cystine. The total sulfate sulfur was still significantly above the fasting level after 12 hours. The organic sulfur increased to a higher level after methionine and

TABLE II

Percentage Changes in Distribution of Sulfur of Plasma Ultrafiltrates after Oral Administration of the Sulfur-Containing Amino Acids

All values are expressed as the average per cent relative to the fasting level values for each experimental animal. The number of experiments averaged is indicated.

Period after amino acid feeding	Cystine fed			Methionine fed		
	Sulfur		No. of experiments	Sulfur		No. of experiments
	Total sulfate	Organic		Total sulfate	Organic	
hrs.	*per cent*	*per cent*		*per cent*	*per cent*	
0.5	113	105	1			
0 75	135	241	1			
1.0	133	206	6	108	325	4
	128*	233*	5*			
3.0	142	204	6	124	345	7
	145*	154*	5*			
6.0	182	140	5	136	360	7
9.0	201	114	1	156	195	1
12.0	134	109	3	139	153	2
3 0†	99	100	6			

* In each of these series there was one value for organic sulfur which deviated greatly from the average value (low in the 1 hour series, high in the 3 hour series) These values represent averages if these two experiments are omitted

† The values in this row are the averages of the glycine control series in which samples were taken 3 hours after glycine was fed in four experiments, and 1 and 4 hours afterward respectively in two experiments.

tended to remain at this high level longer than did the organic sulfur after cystine feeding. A comparison of the changes in the composition of the plasma ultrafiltrates after the ingestion of the amino acids suggested that methionine may be less readily oxidized or utilized than cystine under the conditions of these experiments.

It is possible that differences in the rates of absorption of methionine and cystine from the gastrointestinal canal may be significant here. No comparative data on the rates of absorption in the rabbit are available, so far as is known to us. In the rat, however, the rate of absorption of *l*-cystine, as determined by the Cori procedure, was only slightly higher than that of *dl*-methionine (12). The differences observed may also be related to the fact that the methionine used in the present experiments was the *dl* form while the cystine was *l*-cystine, the naturally occurring isomer. *d*-Cystine (13) is less readily oxidized by the rabbit than is *l*-cystine and is not available for purposes of growth of the young white rat (14). *d*-Methionine, on the other hand, is as effective in growth promotion as is the naturally occurring *l* isomer (15). Stekol (16) has shown that the sulfur of *l*- and of *dl*-methionine is retained equally well by adult dogs maintained on a protein-free diet or by growing dogs fed a low sulfur diet. We are not aware of any published studies in which the rate of excretion of the various sulfur fractions of the urine after methionine administration to rabbits has been determined[2] as after cystine (2). It would be of considerable interest to study changes in the sulfur distribution of plasma ultrafiltrates after the administration of the *d* and *l* isomers respectively of both cystine and methionine.

The cystine values of the ultrafiltrate of fluoride-oxalate plasma (6) of six of the nine animals are given in Table III. It will be noted that the fasting control values were remarkably constant and approximated 1 mg. per cent. When glycine was fed, no changes in the cystine content were observed, thus demonstrating the specific nature of the changes observed when cystine or methionine was administered. When cystine was fed, the values for cystine of the ultrafiltrate of plasma showed a rapid and notable increase and were at a maximum in the samples collected 1 to 3 hours after the administration of the amino acid. The increased cystine sulfur content accounted for most of the increase

---

[2] A comparison of the rates of excretion of the various sulfur fractions of the urine after the administration of *l*-cystine, *l*-methionine, and *dl*-methionine by a human subject is available (17). Cystine as measured by the rate of excretion of sulfate sulfur was less readily oxidized than was either *l*- or *dl*-methionine. The oxidation of *dl*-methionine was "slightly more retarded" than that of *l*-methionine.

in organic sulfur of the filtrates in many cases but this was not invariably true. Thus, with Rabbit 5, the extra organic and cystine sulfur contents were 2.08 and 1.47 mg. respectively 1 hour after cystine feeding and 1.41 and 1.49 mg. in another experiment in which the blood was collected 3 hours after the ingestion of the

TABLE III

Cystine Content of Plasma Ultrafiltrates after Administration of Glycine and the Sulfur-Containing Amino Acids to Rabbits

The descriptive matter at the beginning of Table I should be consulted for details. All substances were administered orally unless otherwise noted. The values are expressed as mg. per 100 cc. of ultrafiltrate

Animal No.	Cystine	Notes	Animal No.	Cystine	Notes
8	1.11	Fasting control	5	1 11	Fasting control
	0 93	" "		1.00	4 hrs. after glycine
	1 09	3 hrs after glycine		6.56	1 hr " cystine
	4 17	1 hr. " cystine		6.67	3 hrs " "
	8 33	3 hrs. " "		2.60	6 " " "
	2.40	6 " " "		16.70*	1 hr. " "
	3 20	6 " " methionine		1 89*	3 hrs. " methionine
6	1.13	3 " " glycine	2	1 25	3 " " glycine
	15.30*	1 hr. " cystine		1.89	1 hr. " methionine
	2.24	6 hrs. " methionine		2.40	3 hrs " "
	1.22	9 5 hrs after methionine		2 60	6 " " "
	3 74	12 hrs. after methionine			
	1.20	12 " " "			
7	1 02	1 hr " glycine	10	1 02	Fasting control
	1 50	1 " " methionine		1 00	" "
	2.95	3 hrs " "		1 91*	1 hr after methionine
	1.70	6 " " "		4 70*	3 5 hrs after methionine

* Subcutaneous injection.

cystine. On the other hand, with Rabbit 8, the corresponding values for extra organic and cystine sulfur were 2.51 and 0.84 mg. after 1 hour and 5.02 and 1.95 mg. after 3 hours. In this latter experiment, as noted in the foot-note to Table II, an unusually high organic sulfur was observed. These differences, which were usually noted in analysis of samples collected shortly after cystine

feeding, suggest the presence of some organic sulfur compound other than cystine in the ultrafiltrates, possibly an oxidation product of cystine. As would be expected, the content of cystine in the ultrafiltrates after subcutaneous injection increased rapidly to high levels (*e.g.*, 15.30 and 16.70 mg., Table III).

After methionine was fed or injected, a significant increase in the cystine content of ultrafiltrates was observed. This increased content although not marked after 1 hour, *e.g.* values of 1.50 and 1.89 mg (Table III), was notable and, we believe, clearly above the error of our experiments in samples taken 3 and 6 hours after the ingestion of the methionine. The highest values observed after oral administration were 3.95 (6 hours) and 3.74 mg. (12 hours). That the increases in cystine content were not related to changes occurring in the gastrointestinal canal was shown with Rabbit 10 (Table III) in an experiment in which the highest cystine value of the methionine series, 4.70 mg. per cent, was observed 3 5 hours after the subcutaneous injection of methionine. In contrast to the results after cystine, the increased cystine sulfur content of the ultrafiltrate did not account for a significant part of the increased organic sulfur. Thus, in the experiment with Rabbit 10 discussed above, the extra cystine sulfur was 1.25 mg. per cent, while the extra organic sulfur was 7.27 mg. per cent. Similar results were observed in all experiments in which methionine was fed. This suggests that, in these instances, the extra organic sulfur of the plasma ultrafiltrates may be chiefly the sulfur of methionine or possibly of some partially oxidized derivative of the amino acid.

The high values for organic sulfur which were believed to be due to the presence of methionine in the ultrafiltrates suggested the desirability of determining whether, under the conditions of our experiments, the presence of methionine would influence the estimation of the small amounts of cystine present. A sample of fasting rabbit blood was divided into two parts and to one was added methionine in an amount somewhat greater than was indicated by the increased organic sulfur of the ultrafiltrates in the feeding experiments. Plasma ultrafiltrates of the two portions showed on analysis 0.90 and 0.93 mg. per cent of cystine respectively. This indicates that the increased cystine values observed in our experiments with methionine are not due to the presence of unchanged methionine in the ultrafiltrates.

These experiments are believed to demonstrate that in the fasting rabbit methionine may be converted into cystine, a demonstration in accord with and in further confirmation of the current belief that cystine may originate biologically from methionine (7–11). Particularly convincing are the experiments of Tarver and Schmidt (10) which were published after the completion of our experiments. It would be desirable actually to isolate the cystine rather than to depend upon a colorimetric reaction as evidence of the increased content of cystine in the plasma. Such isolation involves technical difficulties which are not easy to overcome, particularly when the amounts of cystine involved are as small as in these experiments.

The cystine values obtained by us in plasma ultrafiltrates are lower than those reported by Krijgsman and Bouman (18) for rabbit blood, 10 and 5 mg. respectively 1 to 2 and 3 to 4 hours after the animals had received the *gewohnliches Grunfutter*. No further details are presented. These values seem surprisingly high when compared with the increases observed by us after considerable amounts of cystine were fed.

Since, after the administration of monobromobenzene, cystine is withdrawn from its normal metabolic path and the excretion of conjugated sulfate and mercapturic acid occurs, it was considered of interest to determine the effect of the feeding of this halogen derivative of benzene on the sulfur partition of the plasma ultrafiltrates. Female white rats were fasted 24 hours and the blood was removed from the heart under ether anesthesia. The plasma ultrafiltrates of three control bloods (obtained from groups of four or five rats each) contained 0.11, 0.24, and 0.36 mg. per cent of conjugated sulfate sulfur and 0.92, 0.86, and 0.75 mg. per cent of cystine. Each rat of another group of five animals received a total of 0.5 cc. of monobromobenzene orally in divided doses over a period of 3 days, the animals receiving no food during the 24 hour period immediately preceding the collection of the blood sample. A second group of rats similarly received 1.0 cc. of the monobromobenzene. The conjugated sulfate sulfurs of plasma ultrafiltrates were 0.93 and 0.97 mg. per cent respectively and the values for cystine, 0.09 and 0.21 mg. per cent, values so low as to be beyond the limits of accuracy of the cystine determination. The inorganic sulfate sulfur was within the normal range of values for ultrafiltrates of rat plasma.

SUMMARY

The cystine content and sulfur distribution of ultrafiltrates of the plasma of rabbits which received *l*-cystine and *dl*-methionine orally and subcutaneously have been determined. Methionine appeared to be metabolized somewhat more slowly than cystine, as evidenced by the rate of increase of oxidized (sulfate) sulfur of the plasma ultrafiltrate after the administration of the two amino acids.

After the administration, either orally or subcutaneously, of *dl*-methionine, the cystine content of the plasma ultrafiltrates, as determined by the application of the colorimetric reaction with naphthoquinonesulfonic acid, was increased above the control fasting level. This is in further confirmation of the belief that cystine may be synthesized from methionine in the living organism.

BIBLIOGRAPHY

1. Lewis, H. B , and Brown, B. H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **123**, p lxxv (1938).
2. Stearns, G., and Lewis, H. B , *J. Biol Chem.*, **86**, 93 (1930).
3. Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.*, **104**, 59 (1934).
4 Hele, T. S , and Pirie, N. W., *Biochem. J.*, **25**, 1095 (1931).
5 Pirie, N. W., *Biochem. J.*, **26**, 2041 (1932).
6. Brown, B H , and Lewis, H B., *J. Biol. Chem.*, **138**, 705 (1941).
7. Womack, M , Kemmerer, K. S., and Rose, W. C., *J. Biol. Chem.*, **121**, 403 (1937).
8. Brand, E., Cahill, G. F., and Harris, M M., *J. Biol. Chem.*, **109**, 69 (1935).
9 Lewis, H. B., Brown, B. H , and White, F. R , *J. Biol. Chem.*, **114**, 171 (1936)
10 Tarver, H., and Schmidt, C. L A., *J. Biol Chem.*, **130**, 67 (1939).
11 Beach, E. F , and White, A., *J. Biol. Chem.*, **127**, 87 (1939).
12 Chase, B. W , and Lewis, H. B., *J. Biol. Chem.*, **101**, 735 (1933).
13. du Vigneaud, V , Craft, H A., and Loring, H. S., *J. Biol. Chem.*, **104**, 81 (1934).
14. du Vigneaud, V., Dorfman, R , and Loring, H. S., *J. Biol. Chem* , **98**, 577 (1932).
15 Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1937–38).
16. Stekol, J. A., *J. Biol Chem.*, **109**, 147 (1935).
17. Medes, G , *Biochem. J.*, **31**, 1330 (1937).
18. Krijgsman, B J , and Bouman, E. F., *Arch. néerl. physiol.*, **18**, 458 (1933).

THE DETERMINATION OF ADRENALIN IN BLOOD

By W. R. BLOOR and S. S. BULLEN

(*From the Department of Biochemistry and Pharmacology and the Department of Medicine, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

The chemical method for adrenalin which best fulfils the requirements for its determination in blood is that of Whitehorn (1) as modified by Shaw (2). For the isolation of adrenalin from the blood filtrate Whitehorn used silica which, however, does not give a clean separation and for which Shaw substituted aluminum hydroxide at about pH 8.5 which does adsorb the adrenalin completely. The determination of adrenalin in this method depends on the production by adrenalin of a blue color by the reduction of an arsenomolybdate reagent in strong acid solution. The amount of blue color is greatly increased by the presence of sulfite which, however, also increases the hazards of the method because of the difficulty of controlling the color produced by the sulfite itself. Increasing the concentration of sulfuric acid makes it possible to reduce the color of the blank to a minimum; but the sensitivity to adrenalin is also much reduced, so that for greatest sensitivity it is better to use a procedure which allows some color in the blank. The blue color is given by a variety of substances in blood which are only partly separated from adrenalin by the adsorption procedures used and the present work indicates that many of the figures given in the literature (3–5) for the adrenalin content of blood represent something else.

In the application of Shaw's procedure to the determination of adrenalin in blood the following difficulties were encountered. (*a*) In the first aluminum hydroxide adsorption of the blood filtrate (acid adsorption) 1 drop of 1 N sulfuric acid, as directed by Shaw, was not found sufficient to prevent adsorption of adrenalin. It took 16 to 18 drops of acid and thereby much of the aluminum

hydroxide was dissolved. The hydroxide is a good buffer for either acid or alkali. (b) In the special enhancing treatment described by Shaw in which 0.35 cc. of 4 per cent sodium hydroxide and 2 cc. of water with 2 minutes heating are employed, the aluminum hydroxide did not dissolve completely and the resulting increase of color was small and uncertain. Both these difficulties may possibly be accounted for by differences in the sample of alum used for preparation of the aluminum hydroxide, since Shaw's directions for preparation of the hydrate were carefully followed. In the procedure as finally adopted both these steps were omitted. (c) 5 minutes heating did not give the maximum color, thus adding another factor of uncertainty. It was found by actual measurement of color development (see Fig. 2) that the color increased rapidly for 6 to 8 minutes and after that more slowly, so that a heating time of 10 minutes was used. Tietz, Dornheggen, and Goldman (5), in regard to Shaw's procedure, state, "We have reason to believe that the error in this method as carried out by us is great," and others (personal communications) have had difficulties with the procedure. Both Whitehorn and Shaw found that the control of the blank with sulfite was a serious difficulty in the method and in this we fully agree. The variability of the blank remains one of the major difficulties in the procedure.

Correction for a variable blank color in the ordinary colorimetric matching is not easily made and we decided early that the photoelectric colorimeter, in which the color produced by the substance is automatically corrected by using the blank as the reference solution, offered the best solution of the difficulty. In addition the factors influencing the color in the blank have been studied and changes made, so that difficulty in this direction, while not entirely removed, has been considerably lessened. Changes in procedure have also increased its sensitivity, so that under good conditions amounts of 0.02 γ of adrenalin can be measured with an accuracy of about 5 per cent; that is, the presence of amounts of adrenalin as small as 0.001 γ may be determined. The method, however, remains temperamental—a not unexpected finding considering the minute quantities of material measured—and calls for careful technique with close adherence to a rigid routine throughout the procedure.

The amount of adrenalin in normal blood is not known. Stewart and Rogoff (6), on the basis of many determinations with the biological method of assay, found that the output of the adrenals in cats was about 0.3 to 0.8 γ per kilo per minute, which when distributed in arterial blood on the basis of 80 cc. of blood per kilo of animal would amount to 0.004 to 0.01 γ per cc. Since adrenalin is rapidly removed by the tissues and perhaps also destroyed by the blood, the amount which reaches the venous system would be much smaller. Schlossman (7), also using biological methods, concluded that if present at all adrenalin must be in a concentration of less than 1 part in 1000 million or 0.001 γ per cc. Whitehorn (1) gives a value of 0.025 γ per cc. for cat blood obtained at operation. Shaw (2) gives for rabbit blood 0.05 γ per gm. and for man 0.016 γ per gm. Sarfy (8) using Shaw's procedure found in pigeon blood 0.06 to 0.08 γ per cc. Tietz, Dornheggen, and Goldman (5) found values of about 0.2 γ per cc. in human blood. Giordano and Zeglio (3), using a different method, report values from 1 to 4 γ per cc. in human blood. In the present work, colors corresponding to values of 0.2 to 0.5 γ per cc. of adrenalin were found in venous blood of dogs and humans. This material was, however, found to be stable to alkali under conditions in which adrenalin added to blood or to blood extract was completely destroyed. Blood extracts to which adrenalin has been added, when treated with alkali, give the same or slightly higher values than does the extract containing no adrenalin. This result leaves considerable doubt as to whether there is any adrenalin in venous blood or at any rate amounts greater than about 0.001 γ per cc., which is the limit of measurement of the present method.

Reagents—The reagents used are essentially those employed by Whitehorn and by Shaw.

Arsenomolybdate reagent. Made by dissolving 120 gm. of crystalline sodium molybdate and 20 gm. of crystalline sodium arsenate in 500 cc. of hot water, filtering, cooling, and making up to 1000 cc. 10 cc. of bromine water and 80 cc. of concentrated sulfuric acid are then added. The solution so prepared is unstable, gradually acquires a bluish color, and increases in sensitivity to adrenalin and sulfite until finally it will no longer distinguish between different amounts of material. Protecting it

from light by keeping the solution in a brown bottle greatly slows the change and, if spoiled, the solution may be made useful again by the cautious addition of bromine water. The change in sensitivity is rapid at first, so that if the reagent is used before it is 2 weeks old calibration curves must be adjusted frequently; after that, calibration once a week is necessary.

Sulfuric acid. Four concentrations are used: (a) 1 part of concentrated sulfuric acid to 1 part of water by volume, (b) 1 N sulfuric acid, (c) 0.4 N sulfuric acid, and (d) concentrated sulfuric acid.

Sodium sulfite. A cold, saturated solution made by adding 10 gm. of powdered sodium sulfite (Na_2SO_3) to 25 cc. of boiling water and allowing to cool with occasional shaking. This solution is made fresh each day.

Acid digestion mixture. 5 cc. of the cold, saturated sodium sulfite solution are measured into a 200 cc. Florence flask, 25 cc. of the 1:1 sulfuric acid are added, and the whole well mixed. This solution is made fresh for each set of determinations. It is a disagreeable, fuming mixture and must be handled with care.

Sodium hydrate. Approximately 1 N sodium hydroxide. As ordinarily prepared it contains suspended matter (silica?) which affects the color, especially noticeable in the blank, so that the sample must be allowed to stand for several days or be centrifuged for each day's use and only the clear liquid used. Also, because of its effect on the burette from which it is measured, this instrument must be rinsed out with the solution before use. Impurities in the alkali appear to be an important and perhaps the main factor producing variability in the blanks.

6 N sodium hydroxide (approximately) made by dissolving solid sodium hydroxide in water.

Aluminum hydrate. 100 gm. of potassium aluminum sulfate dissolved in 800 cc. of hot water, filtered, and cooled to 20°. 20 gm. of sodium hydroxide are dissolved in 80 cc. of water, cooled to 20°, and added slowly to the alum solution with continuous stirring. After addition of the alkali, the mixture is well shaken to insure even composition of the hydrate, and then filtered on a Buchner funnel through hardened filter paper. The precipitate is suspended in 800 cc. of distilled water and filtered. The process of washing is repeated twice more, after which the precipitate is

suspended in 400 cc. of water for use, giving a suspension which just does not separate a watery layer and flows freely from a pipette. The suspension has a pH of about 5.3.   Care should be taken to avoid getting fibers of filter .paper into the suspension.   The pipette should be washed free of hydrate after each set of determinations.

Adrenalin standard.   Made by dissolving 50 mg. of adrenalin chloride[1] and 1.25 mg. of alanine in 0.4 N sulfuric acid to make 50 cc.   The first dilution is made by mixing 1 cc. of this standard with 0.4 N sulfuric acid and 2.5 mg. of alanine and making up to 100 cc. (1 cc. = 10 γ of adrenalin).   These two solutions kept in the refrigerator are good for several months.   A dilution of 10 cc. of the second solution plus 2.5 mg. of alanine made up to 100 cc. with 0.4 N sulfuric acid (1 cc. = 1 γ of adrenalin) and a second 1:10 dilution without alanine (1 cc. = 0.1 γ of adrenalin) are kept in the laboratory.   The first of these laboratory solutions must be renewed each month (oftener in hot weather) and the final dilution every week.

Phenolphthalein.   100 mg. in 100 cc. of 0.01 N sodium hydroxide; renewed each week.

Procedure

5 cc. of blood drawn from the vein are run at once into 20 cc. of 10 per cent trichloroacetic acid in a 50 cc. conical centrifuge tube.   After thorough mixing, the material is centrifuged for 5 minutes at about 1000 R.P.M. and the clear fluid poured through a filter into a test-tube or other container.

For determination, 1 cc. of the extract (containing the equivalent of about 0.4 γ of adrenalin) is measured into a small centrifuge tube (16 $\times$ 105 mm.), 1 cc. of 0.4 N sulfuric acid and 2 drops of the phenolphthalein solution added, and the mixture titrated with 1 N sodium hydroxide to a faint pink color.   2 cc. of the well mixed aluminum hydroxide suspension are added and the mixture again neutralized to a faint pink color, after which the material is well shaken (it should now have only the faintest perceptible pink tint), allowed to stand for 5 minutes, and centrifuged for 5 minutes at about 2000 R.P.M.   The supernatant fluid is poured

[1] Kindly supplied by Parke, Davis and Company.

off, the tubes drained, and the liquid completely removed from the end of the inverted tube with absorbent paper (the amount of water in the reaction mixture is important). 2 cc. of the arseno-molybdate reagent are added and the precipitate well mixed with it. The mixture is set in boiling water for 5 minutes and then to the clear solution are added 3 cc. of the acid digestion mixture of 1:1 sulfuric acid and sodium sulfite and the digestion in the boiling water continued for 10 minutes, after which the tube is set in ice water (with an excess of ice) for 15 minutes. The solution is then diluted to 40 cc. and the color measured in 10 cc. of this dilution. A blank determination on the reagents alone is run along with the samples. Determinations are carried out in quadruplicate and the average taken as the value. It is emphasized that measurements of time and volume must be exact and the routine strictly adhered to.

Colorimeter—The measurement of the color was made by a photoelectric colorimeter which was an adaptation of that described by Eddy and DeEds (9). The modifications consisted in (1) the use of the Evelyn (10) light system employing a flashlight bulb, focusing device, and a 6 volt storage battery; (2) slots in the inside of the box carrying bakelite plates fitting into the slots, holding in order from below (a) the light system, (b) the filter (Corning, Signal Red), (c) a sliding cup carrier, working between guides and controlled from outside the box by a short rod, with places for two colorimeter cups (or a block suitably perforated to carry a small test-tube), and (d) the barrier layer photoelectric cell connected with a galvanometer.

Readings—The colored solution, after the 15 minutes chilling, was diluted to 40 cc. and 10 cc. were measured into a colorimeter cup. The blank solution was similarly diluted and 10 cc. measured into another colorimeter cup matched to the first one or adjusted to it. The cups were placed in the colorimeter, the cup with the blank solution in the left-hand position. With the blank cup in the path of light, adjustments of the rheostats on the light circuit were made until the indicator on the galvanometer read some convenient figure, ordinarily 20 (200 divisions) or its equivalent to match 20 on the other cup. The cup with the unknown was then moved into position and the reading taken. The difference between the 200 reading of the blank and the reading of the un-

known represents the light absorption of the latter. Its value in terms of adrenalin was then read off the calibration curve (see Fig. 1) and by calculation the value of the original sample was determined. In this way, correction for the blank was made automatically. The amount of colored solution used for the reading contained one-fourth of the total color obtained from 1 cc. of extract, which was one twenty-fifth of the amount from 5 cc. of blood. The values obtained were then multiplied by 100 to obtain the value for 5 cc. of blood. From this it may be seen that if desirable much smaller amounts of blood may be used.

Calibration Curve—A calibration curve was made as follows: Samples of standard adrenalin solution containing from 0.4 to 1.2 γ were measured into the round bottom centrifuge tubes used in the method; 1 cc. of the 10 per cent trichloroacetic acid and 2 drops of the phenolphthalein were added and the neutralization, adsorption on aluminum hydroxide, color development, and readings carried out exactly as described. The galvanometer readings obtained were subtracted from 200 and the values of light absorption plotted against adrenalin values, as in Fig. 1. Since the colored solution was diluted to one-fourth its volume, the values plotted are one-fourth of those of the samples taken.

Development of the Color—In order to determine the optimum time of heating in boiling water for the development of the characteristic color, experiments were made as follows, with the test-tube adapter in the photoelectric colorimeter. A sample of adrenalin solution of known content was carried through the regular procedure to the end of the 5 minute heating with the arsenomolybdate reagent. The 1:1 sulfuric acid was then added, and the whole well mixed and placed in the photoelectric colorimeter which was then adjusted to a convenient value, ordinarily 200 scale divisions on the galvanometer. The tube containing the mixture was then set in the boiling water and at intervals of 2 minutes thereafter removed and readings made. A blank of the reagents was similarly treated throughout. Characteristic results in the form of curves are shown in Fig. 2, from which it is seen that the rate of color development is rapid at first, the maximum color and the maximum difference between blank and test coming at about 6 to 8 minutes. After that the increase in color was slow and was about equal in the test and in the blank

up to 18 minutes. The time selected for the color development was therefore 10 minutes.

Placing the tubes in ice water for 15 minutes increases the depth of color and the amount of increase of color appears to depend con-
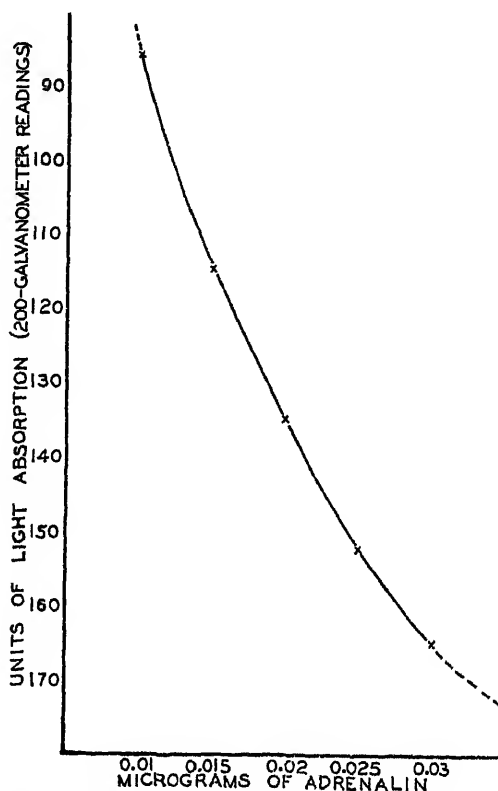
FIG. 1. Calibration curve; units of light absorption by micrograms of adrenalin.

siderably on the temperature of the cooling water, hence the need of keeping ice in the water during the cooling period.

Effect of Alkali—Alkali destroys adrenalin, while it has no (or a slight enhancing) effect on the other substances present in blood which give color in this reaction. The test is carried out as follows: The procedure as outlined above is carried to the stage

of draining the supernatant liquid from the aluminum hydroxide after the centrifugation. To the centrifuged precipitate in the tube is added enough 6 N alkali just to dissolve it, generally 5 to 6 drops. Solution is aided by stirring with a glass rod and the tube with the dissolved material is immersed in boiling water for 3 minutes, cooled by a short immersion in ice water, and made just
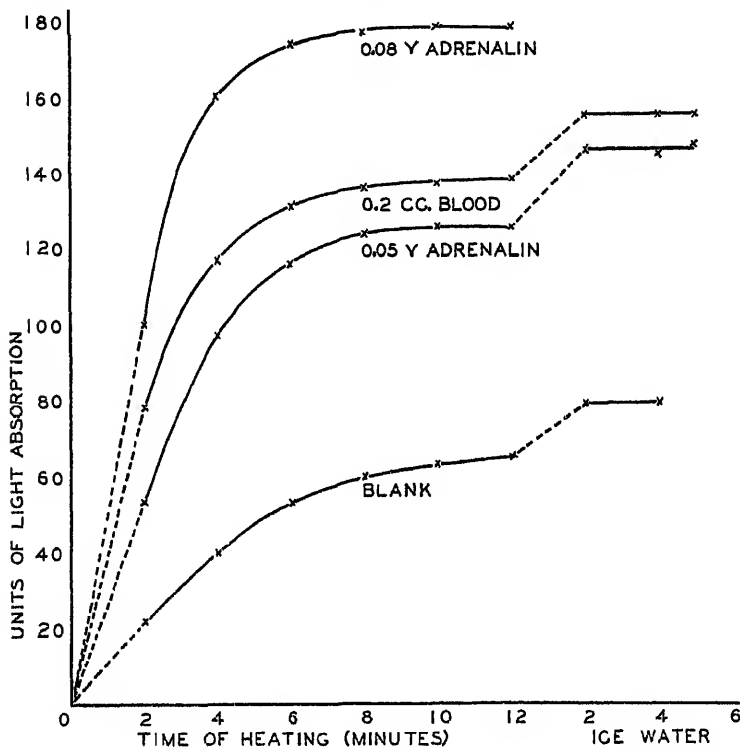
FIG. 2. Development of color with time by adrenalin on acid arseno-molybdate in boiling water (99°).

acid (to phenolphthalein) with concentrated sulfuric acid. These strong solutions are used to keep down the volume of water, which strongly affects the color production. After neutralization, which results in a white salty mass, the 2 cc. of arsenomolybdate reagent are added, the mixture heated for 5 minutes, and the color production carried out as before. In known adrenalin solutions

the alkali treatment reduces the color production to that of the blank. The color production of blood extracts is increased about 10 per cent by the alkali treatment and is the same whether adrenalin was present or not. The alkali treatment of blood extracts thus serves to distinguish adrenalin from the other substances which give the color under the conditions of the determination.

The demonstration of adrenalin in blood thus requires two determinations, one with and one without the use of alkali. If the result with alkali is higher than that without, adrenalin is absent from the sample. If it is lower, then the amount of adrenalin present is the difference between the two values corrected by the 10 per cent by which the values for the blood reducing substances are elevated by the alkali treatment. Up to the present it has not been possible to demonstrate adrenalin in either dog or human venous blood.

By the procedure described amounts of adrenalin of 0.015 to 0.25 γ can be measured with an accuracy of about 5 per cent; i.e., amounts of adrenalin as small as 0.001 γ or 1 part in 1000 million of solution will be shown.

To obtain this sensitivity and accuracy, it is necessary that quantitative care be taken at every step in the procedure and if departures from the directions given for time and amount are made it should be only after one has made sure that the changes do not affect the values.

Results

Recovery of adrenalin added to trichloroacetic acid extracts of blood was found to be practically complete (95 per cent or better), as reported by Shaw. Shaw also stated that recovery from blood, while good, was not complete. By the present procedure recovery of adrenalin added to freshly drawn dog blood was found to be as complete as from blood extracts. Experiments were made as follows: A known amount of a standard adrenalin solution in 0.4 N sulfuric acid (from 0.7 to 2.0 γ) was measured into a small test-tube, a drop of the aqueous phenolphthalein solution added, and the mixture titrated just to alkalinity with 1 N sodium hydroxide. 1 N sulfuric acid was immediately added to bring the mixture to faint acidity. A blank solution containing the same

amount of acid without adrenalin was similarly treated.   About 12 cc. of blood were drawn from the jugular vein of a dog and 5 cc. samples measured at once into the two test-tubes.   After mixing, the blood was decanted into 50 cc. conical centrifuge tubes containing 25 cc. of 10 per cent trichloroacetic acid and mixed; the test-tubes were rinsed out, the mixture was well

TABLE I

Recovery of Adrenalin from Blood*

Dog No.	Adrenalin added	Adrenalin recovered	
	γ	*γ*	*per cent*
40-56	0.75	0 756	101
40-56	1 6	1.8	112
40-56	1 6	1 36	85
38-101	1 2	1 18	98
40-2	1 8	1 6	90
39-273	1.0	0.95	95
39-273	1.4	1.23	90
38-346	1.5	1.5	100
38-101	2 0	1.93	97
38-346	1 5	1.40	93
39-273	1.5	1.43	95
38-101	1.5	1.55	103
39-273	1.2	1.11	93
39-25	1 5	1.5	100
38-101	1 2	1.17	98
38-346	1.5	1.4	93
36-26	1.5	1 55	103
38-101	1.6	1.5	94

* The adrenalin values decrease on standing.   Thus, in Dog 38-346 above (immediate recovery of 93 per cent), the recovery 3 hours later was 55 per cent and 35 per cent the next day

shaken, centrifuged, and the clear filtrate used as in the procedure described above.   Recovery is shown in Table I.

The adrenalin-like reducing substances in blood were determined for a number of dogs and a few humans.   The values obtained varied from 0.24 to 0.36 γ per cc. in both   This substance is both acid- and alkali-stable.   Tests made on human urine showed the presence of a substance which behaved similarly to the reducing substance in blood and was present in about 10 times the concen-

tration found in blood. It was alkali-stable and was not affected by hydrolysis with sulfuric acid.

The method has been applied to the determination of adrenalin in commercial samples with the following results, expressed in mg. per cc.

Manufacturer No.	Sample No.	Found	Claimed
I	I	1 10	1.0
I	II	1 00	1.0
I	III	1.06	1.0
II	I	0 87	1.0

Samples I and II of Manufacturer I kept in cork-stoppered bottles for about a year were found to be brownish in color and to have values of 0.72 mg. per cc. (Sample I) and 0.7 mg. per cc. (Sample II).

Recovery of adrenalin injected directly into the circulation has been tried a few times. After a control blood sample was taken from a dog, a known dose of adrenalin, 0.25 to 0.8 mg., was injected into the jugular vein of one side and samples were taken at intervals from the vein on the other side. Measured quantities of the blood were run at once into 4 volumes of 10 per cent trichloroacetic acid, mixed by shaking, centrifuged, and determinations made on the filtrate. The difference in values between the control and the sample was taken as representing adrenalin, an assumption which was supported by the fact that alkalization of control and sample gave in all cases the same values.

A typical experiment was as follows: 0.77 mg. of adrenalin (48 γ per kilo of body weight) was given; preliminary control sample 0.208 γ per cc.; 1 minute after injection 0.546 γ per cc.; 10 minutes after injection 0.377 γ per cc.; 20 minutes after injection 0.213 γ per cc.

If 80 cc. of blood per kilo of animal (a total of 1520 cc.) is allowed, the amount of adrenalin present in the blood was 0.514 mg. or 66 per cent of the dose 1 minute after injection, 0.257 mg. or 33 per cent of the dose 10 minutes after injection, and a trace 20 minutes after injection.

SUMMARY

A procedure is described for the determination of adrenalin in blood by which amounts of 0.02 γ can be measured with an accuracy of about 5 per cent.

A means is provided for distinguishing adrenalin from other similarly reacting substances in blood.

With this method, it was found that, if present at all, the adrenalin content of venous blood of man and dog is less than 0.001 γ per cc. or 1 part in 1000 million.

The substances in blood which react similarly to adrenalin are present in amounts of about 0.25 γ per cc.

BIBLIOGRAPHY

1. Whitehorn, J. C., *J. Biol. Chem* , **108**, 633 (1935)
2 Shaw, F. H., *Biochem. J.*, **32**, 19 (1938).
3 Giordano, C , and Zeglio, P., *Arch sc med* , **64**, 325 (1937)
4. Bloor, W. R., *Proc. Am Soc. Biol. Chem.*, *J. Biol. Chem.*, **128**, p. ix (1939).
5. Tietz, E. B., Dornheggen, H., and Goldman, D , *Endocrinology*, **26**, 641 (1940)
6. Stewart, G. N., and Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, **10**, 1 (1917).
7. Schlossman, H , *Arch exp Path. u. Pharmakol* , **121**, 160 (1927).
8. Sarfy, E., *Z physiol. Chem.*, **262**, 87 (1939)
9 Eddy, C. W , and DeEds, F., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 12 (1937).
10. Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).

.

THE DETERMINATION OF SOME AMINO ACIDS IN TOBACCO MOSAIC VIRUS PROTEIN

By A. FRANK ROSS

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*)

Several plant viruses have been isolated in the form of characteristic nucleoproteins of high molecular weight (1-5). The properties of many of these have been determined and compared, but with few exceptions (6, 7) analytical data have been limited to elementary analyses and to determinations of the amount and kind of nucleic acid present. Comparatively little is known of the protein components which comprise the greater part of the virus molecules. A better understanding of the chemical structure that imparts to viruses the ability to reproduce and to mutate, properties also characteristic of accepted living organisms, would obtain if the components of which the different viruses are built were known. It is important to know the number and kind of reactive groups present in different types of virus molecules, the manner in which viruses resemble one another in composition and how they differ, and the respects in which they differ from other proteins. Since many viruses mutate, giving rise to strains which are closely related proteins, analytical data on such proteins might relate changes in biological, chemical, serological, and physical properties to changes in composition. It is possible that specificity of host range (8) may be explained by the ability or inability of a particular species of plant to provide certain amino acids required by a given virus. In this paper are reported some of the analyses for amino acids that have been made on tobacco mosaic virus protein. While a complete analysis of the protein component has not been obtained, a fair proportion of the molecule has been accounted for and some information concerning the nature of the remainder has been obtained. It is recognized that some of the

values presented may differ somewhat from values that may be obtained in the future by improved methods (9). However, the data make possible a comparison with other proteins at the present time, and it is hoped that they will constitute a basis for a comparative examination of other strains of this virus and of other viruses. A preliminary report of part of this work has previously (10) been given.

Preparation of Material—The preparations previously described (6) were used in most instances, although during a part of the work additional samples were prepared by the same methods. All preparations were dried to a moisture content of about 8 per cent by freezing and drying *in vacuo*. All determinations were based on samples dried to constant weight at 110°. Unless otherwise specified, a Goudsmit-Summerson photoelectric colorimeter manufactured by the Klett Manufacturing Company, Inc., was used.

Tyrosine and Tryptophane—The Folin-Marenzi (11) method was found unsatisfactory, for cloudy solutions were obtained. Lugg's (12) modification of the method gave reproducible results. Virus samples weighing 200 to 300 mg. were hydrolyzed as described by Bailey (13) and aliquots containing 1 to 2 mg. of tyrosine were taken for comparison. A few determinations were made by means of Bernhart's (14) micromethod for tyrosine and Shaw and McFarlane's (15) glyoxylic acid method for tryptophane. In the last named method, 50 mg. of virus were dissolved in 1 cc. of 20 per cent sodium hydroxide. This solution was made to 25 cc. and an aliquot portion removed for color development and comparison with a tryptophane standard. In the Bernhart and glyoxylic acid methods, a Klett-Summerson photoelectric colorimeter with a green filter was used. The results are presented in Table I. A tyrosine content of 3.9 per cent was indicated by both methods. In the case of tryptophane, a given method gave reproducible results, but the Folin-Marenzi-Lugg method gave lower results than did the other procedure. The discrepancy may be due to losses during hydrolysis, to the fact that the color fades quite rapidly, or to the presence of an interfering substance in this particular protein. Shaw and McFarlane consider that tryptophane combined in a protein gives the same amount of color as

does the free acid, but it is possible that in some combinations the color formation may be enhanced. However, for the present, the higher value of 4.5 per cent will be regarded as more nearly correct.

Arginine—Arginine was determined by Weber's (16) modification of the Sakaguchi (17) reaction. The protein was hydrolyzed by boiling for 20 hours with 20 per cent HCl; humin was removed by filtration and then well washed. Aliquot portions of the combined filtrate and washings were diluted and aliquots containing

TABLE I

Tyrosine and Tryptophane Content of Tobacco Mosaic Virus

Amino acid	Method	Amount in virus*		
		Sample 1†	Sample 2‡	Sample 3†
		per cent	*per cent*	*per cent*
Tyrosine	Folin-Marenzi-Lugg	3 9	3.9	
		3 9	3 9	
		3 7	3 8	
	Bernhart's micromethod	3.8§	3.9§	3 9§
		3 8	3.8	3.9
Tryptophane	Folin-Marenzi-Lugg	1 9	2.0	
		2.1	2.0	
		2.0	2.1	
	Glyoxylic acid method	4.6§	4.5§	4.4§
		4 5	4.5	4.5

* The figures in these columns are the averages of two or more analyses of a hydrolysate or solution.

† Ultracentrifugally isolated.

‡ Chemically isolated

§ Dr. C A. Knight of this laboratory has recently obtained similar results with these and with other samples of tobacco mosaic virus.

0.004 to 0.01 mg. of arginine were taken for the determinations. The results, which are presented in Table II, indicate an arginine content of 9.0 per cent. Arginine was precipitated from other hydrolysates by the method of Van Slyke (18) and the precipitate dissolved in dilute alkali. Aliquot portions of this were taken for analysis. The remainder was freed of phosphotungstic acid by the method of Van Slyke and the resulting solution also analyzed for arginine. The results were only slightly lower than those obtained with the diluted hydrolysates. The value here reported

has also been confirmed by the isolation of arginine by means of flavianic acid. The details will appear in a later paper.

Histidine—The fractions described above that were used for arginine determinations were also. used for tests for histidine. Jorpes' (19) modification of the Pauly (20) reaction and the Kapeller-Adler (21) test as modified by Woolley and Peterson (22) both gave negative results with one exception. In that one case, sufficient tyrosine was found to be present to give the test. An amount

TABLE II

Arginine and Histidine Content of Tobacco Mosaic Virus

Amino acid	Method	Fraction used for determination	Amount found*
			per cent
Arginine	Sakaguchi-Weber	Acid hydrolysate diluted	9.0
			9.1
			9.1†
			9.1†
		Phosphotungstic acid ppt.	8 9
		Decomposed phosphotungstic acid ppt.	8.9
Histidine	Pauly-Jorpes	Phosphotungstic acid ppt.	0.0
			0.04‡
		Decomposed phosphotungstic acid ppt.	0.0
			Trace‡
	Modified Kapeller-Adler	" "	0.0

* Each figure in this column is the average of two or more determinations.

† An ultracentrifugally isolated sample was used for analysis. In all other cases, a chemically isolated sample was used.

‡ Probably due to the presence of a trace of tyrosine.

of histidine equivalent to less than 0.1 per cent of the protein could be recovered when added to the fractions. Hence, it appears that histidine is not present in the tobacco mosaic virus molecule.

Phenylalanine—Phenylalanine was determined by Block's (23) modification of the Kapeller-Adler (24) reaction. The test was applied directly to acid hydrolysates and also to the monoamino acid fraction obtained by the Van Slyke (18) procedure. The two methods gave somewhat different values, as may be seen in Table III. The values are probably only approximations, for the

intensity of color development is influenced by other amino acids. Block added glycine to standards, a procedure which caused enhanced color development. In the present work, the addition of leucine resulted in an inhibition of color formation. The values reported were obtained with controls to which no amino acid other than phenylalanine was added.

Serine—Samples of the virus protein were hydrolyzed by boiling with 20 per cent HCl for 20 hours. The excess HCl was removed

TABLE III

Determination of Some Amino Acids in Tobacco Mosaic Virus

Amino acid	Method	Fraction used for determination	Amount found*
			per cent
Phenylalanine	Block	H_2SO_4 hydrolysate	6.6
			6.7†
		Van Slyke monoamino acid fraction	5.6
Serine	Rapoport	" "	6.4
			6.5†
			5.7
Glycine	o-Phthalaldehyde	Acid hydrolysate	None
	Potassium trioxalatochromiate	" "	"
Threonine	Block-Bolling	" "	5.2†
			6 2
			4 6
Proline	Bergmann's solubility method	" "	4 67
			4.66†

* Each figure in this column represents the average of two or more analyses of a hydrolysate.

† Ultracentrifugally isolated sample. All others were chemically isolated.

from the hydrolysates by repeated evaporation under reduced pressure. The basic amino acids were removed by precipitation with phosphotungstic acid (18) and the dicarboxylic acids by the method of Jones and Moeller (25). The solutions were freed of barium, then analyzed for serine by the method of Rapoport (26) in which serine is deaminated, and then condensed with naphthoresorcinol. The results indicate that the protein is unusually rich in serine; if the method is assumed to be accurate and glycine

is in fact not present, the presence of about 6.4 per cent serine is suggested.

Glycine—The *o*-phthalaldehyde reaction of Zimmermann (27) was used for the estimation of glycine. The protein was hydrolyzed and the test applied as described by Patton (28). No chloroform-soluble color developed, although the test was repeated several times. When an amount of glycine equivalent to less than 0.5 per cent of the protein was added to the hydrolysate, a chloroform-soluble color resulted, but it did not exactly match the standard. The results are indicative, but not proof, of the absence of glycine in the virus protein.

Potassium trioxalatochromiate was also used in several attempts to precipitate glycine by the method described by Bergmann and Fox (29). In a typical experiment, 9.8 gm. of chemically isolated protein were hydrolyzed by boiling 20 hours with 20 per cent HCl. The hydrolysate was freed of excess HCl by repeated evaporations under reduced pressure, then adjusted to pH 2 with silver oxide. The silver chloride was washed with hot water and the combined washings and filtrate concentrated to 26 cc. To this, 6.2 cc. of 10 per cent HCl, 2 gm. of potassium trioxalatochromiate, and 46 cc. of absolute alcohol were added. The mixture was shaken for 8 hours, then placed in a cold room overnight. The precipitate, when collected on a filter, washed with a cold 3:1 mixture of absolute alcohol and 0.5 N HCl, and dried in air, weighed 0.7397 gm. However, it contained but 0.14 per cent amino nitrogen, corresponding to less than 0.1 per cent glycine in the protein. Under comparable conditions, 78 per cent recovery was obtained with 100 mg. of glycine. The experiment was repeated several times, with smaller amounts of the reagent as well as different concentrations of hydrolysate, and similar results were obtained. The amount of amino nitrogen in the precipitate was always quite low; hence it seems probable that it was due to small quantities of other amino acids adsorbed on the excess reagent that precipitated. When a quantity of glycine equivalent to about 1 per cent of the protein was added to a hydrolysate, most of it could be recovered. The data indicate that glycine is not present in the virus protein.

Threonine—Threonine was determined by the method of Block and Bolling (30). Considerable variation in results was encountered, as may be seen in Table III. The values obtained are

probably a rough estimate of the amount of threonine in this protein, but it is possible that acetaldehyde may have arisen from other sources.

Hydroxyproline and Proline.—The reaction for hydroxyproline was applied according to the directions of McFarlane and Guest (31), except that hydrolysis was accomplished by boiling for 20 hours with 20 per cent HCl. Negative results were obtained in all cases with both chemically and ultracentrifugally isolated samples. However, when hydroxyproline was added to the hydrolysates, the test was still negative. The procedure was varied in many ways and a partial separation of hydroxyproline from other amino acids was made, but the results were invariably negative. Hence, this test is not applicable to tobacco mosaic virus hydrolysates. Proline was determined by the method of Guest (32), with hydrolysates prepared for the hydroxyproline tests. The results indicated about 8 per cent proline. However, this value is higher than that obtained by other means, and since hydroxyproline also gives a positive test, it appears probable that the latter amino acid is present in the virus molecule.

Bergmann and Stein's (33) solubility method was also utilized in the determination of proline. In one experiment, 11.48 gm. of the chemically isolated virus were hydrolyzed by boiling 18 hours with 20 per cent HCl. The hydrolysate was freed of excess HCl and decolorized as described by Bergmann and Stein. It was then concentrated under reduced pressure, 1 cc. of concentrated HCl was added, and the solution adjusted to 100 cc. 20 cc. portions of methyl alcohol containing 0.3584, 0.4578, and 0.5824 gm., respectively, of ammonium rhodanilate[1] were added to three separate samples each of 32 cc. of the hydrolysate. The flasks were stoppered, allowed to stand 16 hours at 0°, then shaken for 4 hours at 4°, and again allowed to stand for 24 hours at 0°. The precipitates were collected by filtration at 0° on sintered glass funnels, washed with 4 cc. of ice-cold water, and then dried in air and finally to constant weight over $CaCl_2$ and NaOH. When calculated by the method of Bergmann and Stein, values of 4.74, 4.64, and 4.64 per cent, average 4.67 per cent, were obtained. The results from a similar experiment averaged 4.66 per cent.

[1] The reagent was prepared as described by Bergmann (34) and repurified before use by the method of Bergmann and Stein (33).

DISCUSSION

The significance of amino acid analyses is dependent, of course, upon the purity of the protein.   The fact that no differences were detected between the chemically isolated samples and those isolated by means of the ultracentrifuge indicates that the samples were essentially pure.   If significant quantities of impurities were present in one type of sample, it seems probable that the same kind and quantity would not be contained in a sample isolated by an entirely different method.   Furthermore, considerable evidence has accumulated indicating that ultracentrifugally isolated virus protein approaches a high degree of homogeneity and contains no significant quantities of impurities.

Since the tobacco mosaic virus molecule has a molecular weight of about 43 million (35), there is little to be gained by calculating the frequencies of the amino acid distribution by the method of Bergmann and Niemann (36).   The suggestion has been made by Astbury (37) that the virus may be made up of uniform subunits.   If this is true, the smallest possible uniform unit, based on a cysteine content of 0.68 per cent (6), would correspond to a molecular weight of about 17,800, a unit considerably larger than that suggested by Astbury.   Of the several determinations reported in this paper, those for arginine, tryptophane (glyoxylic acid method), and for tyrosine gave results in fair agreement with Bergmann and Niemann's hypothesis based on the above subunit.

SUMMARY

Tobacco mosaic virus has been found to contain 3.9 per cent tyrosine, 4.5 per cent tryptophane, 4.7 per cent proline, 9.0 per cent arginine, 6 7 per cent phenylalanine, 6.4 per cent serine, and about 5.3 per cent threonine.   Glycine and histidine appear to be absent.   No differences were noted between chemically isolated samples and those isolated by means of the ultracentrifuge.

BIBLIOGRAPHY

1. Stanley, W. M., *Science*, **81**, 644 (1935); *Phytopathology*, **26**, 305 (1936); *J. Biol Chem* , **117**, 325 (1937); **129**, 405 (1939).
2. Bawden, F. C., and Pirie, N W , *Proc. Roy Soc. London, Series B*, **123**, 274 (1937); *Brit. J. Exp. Path.*, **19**, 66, 251 (1938), **20**, 322 (1939).
3. Loring, H. S., *J. Biol. Chem.*, **121**, 637 (1937).

4. Pirie, N. W , Smith, K. M., Spooner, E. T C., and McClement, W. D., *Parasitology*, **30**, 543 (1938).
5. Ross, A. F., *Phytopathology*, in press (1941).
6. Ross, A F., *J. Biol. Chem*, **136**, 119 (1940).
7. Schramm, G , and Muller, H., *Naturwissenschaften*, **28**, 223 (1940).
8. Holmes, F. O., *Phytopathology*, **28**, 58 (1938).
9. Rittenberg, D , Keston, A S., Rosebury, F , and Schoenheimer, R., *J. Biol. Chem*, **127**, 291 (1939).
10. Ross, A. F., and Stanley, W. M., *Proc. Am. Soc. Biol. Chem*, *J. Biol. Chem*, **128**, p. lxxxiv (1939).
11. Folin, O., and Marenzi, A. D., *J. Biol. Chem*, **83**, 89 (1929)
12 Lugg, J. W H., *Biochem. J.*, **31**, 1422 (1937).
13. Bailey, K , *Biochem. J.*, **31**, 1406 (1937)
14. Bernhart, F W., *Proc Am. Soc. Biol. Chem.*, *J Biol. Chem.*, **123**, p. x (1938).
15. Shaw, J L D., and McFarlane, W. D , *Canad. J. Research, Sect. B*, **16**, 361 (1938)
16. Weber, C J , *J Biol Chem*, **86**, 217 (1930).
17 Sakaguchi, S., *J. Biochem.*, Japan, **5**, 25 (1925).
18. Van Slyke, D. D., *J. Biol. Chem*, **10**, 15 (1911–12)
19 Jorpes, E , *Biochem. J*, **26**, 1507 (1932).
20. Pauly, H., *Z. physiol. Chem*, **42**, 508 (1904).
21 Kapeller-Adler, R , *Biochem Z*, **264**, 131 (1933).
22 Woolley, D. W , and Peterson, W H , *J. Biol Chem.*, **122**, 207 (1937–38).
23 Block, R J , The determination of the amino acids, Minneapolis, revised edition (1938).
24. Kapeller-Adler, R , *Biochem Z*, **252**, 185 (1932).
25. Jones, D B , and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).
26 Rapoport, S , *Biochem. Z.*, **281**, 30 (1935).
27. Zimmermann, W., *Z. physiol. Chem*, **189**, 4 (1930).
28. Patton, A. R., *J. Biol. Chem.*, **108**, 267 (1935).
29. Bergmann, M., and Fox, S. W., *J. Biol. Chem*, **109**, 317 (1935).
30. Block, R. J , and Bolling, D., *J. Biol. Chem.*, **130**, 365 (1939).
31. McFarlane, W. D., and Guest, G. H., *Canad J. Research, Sect. B*, **17**, 139 (1939).
32. Guest, G H., *Canad. J. Research, Sect. B*, **17**, 143 (1939).
33. Bergmann, M., and Stein, W. H., *J. Biol. Chem.*, **128**, 217 (1939).
34. Bergmann, M , *J. Biol. Chem.*, **110**, 471 (1935).
35. Lauffer, M A , *J. Biol. Chem.*, **126**, 443 (1938)
36 Bergmann, M., and Niemann, C , *J. Biol Chem*, **115**, 77 (1936).
37. Astbury, W. T., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 359 (1938).

.

"COUPLING" OF PHOSPHORYLATION WITH OXIDATION OF PYRUVIC ACID IN BRAIN*

By SEVERO OCHOA†

(From the Department of Biochemistry, Oxford University, Oxford, England)

An analysis of the dialyzable components of the pyruvic acid oxidation system of brain showed that in addition to cocarboxylase (thiamine pyrophosphate) (2), a C_4 dicarboxylic acid (2), and magnesium or manganese ions (3), both inorganic phosphate and "adenine nucleotide" (adenylic acid, adenosine triphosphate) (4) are required. The latter observation suggested that a cycle of phosphorylation is involved in the enzymatic oxidation of pyruvate by animal tissues. With acetone-dried preparations of *Bacillus delbruckii* Lipmann had found that dehydrogenation of pyruvic acid required both cocarboxylase and inorganic phosphate and that, upon addition of adenylic acid, inorganic phosphate disappeared with an equivalent formation of adenosine polyphosphate (5). The two lines of research show that phosphorylation processes take a part in the enzymatic oxidation of pyruvic acid generally. The present research was undertaken to study the relationship between phosphorylation and oxidation of pyruvate in brain.

Though esterification of inorganic phosphate with either adenylic acid or creatine accompanies oxidation of pyruvate in brain preparations, its direct demonstration is difficult owing to the presence of adenosine triphosphatase (4). Esterification may be demonstrated, however, if in the presence of catalytic amounts of adenylic acid either hexose monophosphate or glucose is used as phosphate acceptor (1). Aerobic phosphorylation of glucose, and of other P acceptors, has been investigated by Kalckar in kidney preparations (6, 7), and more recently by Colowick, Welch,

---

and Cori (8). This phosphorylation, which did not seem to involve a participation of the adenylic acid system, was believed to be coupled either with the oxidation of a number of substrates not including pyruvate (6), or with that of a dicarboxylic (probably succinic) acid (8). Colowick *et al.* (8) also found that oxidation of pyruvic acid catalyzed by C_4 dicarboxylic acids produced phosphorylation of glucose in kidney extracts.

I have found that no more than half of the esterification of inorganic phosphate with either hexose monophosphate or glucose, occurring in brain dispersions when pyruvate is oxidized (with fumarate as catalyst), can be related to oxidation of a dicarboxylic acid; the rest is connected with oxidation of pyruvate itself. Evidence has also been obtained to support the view that oxidation of pyruvate is connected with phosphorylation of adenylic acid to adenosine polyphosphate which transfers its labile phosphate groups to either hexose monophosphate or glucose with formation of hexose diphosphate.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods*</div>

Pigeons, either normal or vitamin B_1-deficient, were used throughout this work. Brain dispersions were prepared and dialyzed at 0–3° as previously described (2). In most cases 1.5 cc. of the dialyzed enzyme[1] were measured into ice-cold Warburg bottles containing the remaining additions to make a volume of 2.2 to 2.5 cc. Additions always included Na_2HPO_4-KH_2PO_4 buffer, pH 7.3, and Mg^{++} as $MgCl_2$. In all cases the reaction mixtures were deproteinized when required by tipping in 0.2 cc. of 40 per cent trichloroacetic acid from the side bulbs. The trichloroacetic acid mixtures were centrifuged, and aliquots of the clear centrifugate were used for the various estimations.

Orthophosphate was determined colorimetrically, as in previous experiments (4), and the various phosphate ester fractions after conversion to orthophosphate by the methods developed by the Meyerhof school. In a few experiments bisulfite-binding substances were estimated by the method of Clift and Cook (9), and the results expressed as pyruvic acid after correction for the triose phosphate present. Fructose was also determined in some

---

[1] Equivalent to approximately 300 mg. of fresh brain.

cases by Roe's modification (10) of Seliwanoff's method; a solution of the sodium salt of fructose-1,6-diphosphate (concentration checked by P estimation) was used as standard, and the results were expressed in terms of hexose diphosphate P.

Freshly made up solutions of crystalline sodium pyruvate were used as in previous work (4). Cocarboxylase was a synthetic specimen (65 per cent purity) kindly supplied by Merck and Company, Inc. Hexose monophosphate was prepared in this laboratory, by Mr. L. A. Stocken, by the method of Smythe (11),[2] and phosphoglycerate from yeast extract by the method of Vercellone and Neuberg (12). Phosphopyruvate was a synthetic specimen kindly supplied by Professor O. Meyerhof. All other substances were commercial preparations. The barium salts were converted into the sodium salts before use.

Phosphorylation of Adenylic Acid and Creatine

When dialyzed brain dispersions are incubated aerobically with phosphate, magnesium, fluoride, and a few mg. of either adenylic acid or creatine, no change in the concentration of inorganic phosphate takes place, but a small disappearance is observed when pyruvate and fumarate are also added. This disappearance is accompanied by an approximately equivalent formation of easily hydrolyzed phosphate (7 minutes at 100° in 1.0 N HCl) when the P acceptor is adenylic acid, or phosphocreatine when creatine (in the presence of catalytic amounts of adenylic acid) is the P acceptor (Table I). The experiments of Table I were carried out by shaking the samples in air, at 28°, in conical flasks of 50 cc. capacity. At various times the mixtures were deproteinized with 2.5 cc. of ice-cold 5 per cent trichloroacetic acid, cooled in ice for a few minutes, and filtered. For the phosphocreatine estimations aliquots of the filtrate were immediately treated with ice-cold alkaline magnesia mixture to precipitate the true inorganic phosphate.

It will be observed (Table I, Experiment 3) that in the absence of fluoride the esterification is significantly smaller. The effect of fluoride will be discussed later in connection with the phosphorylation of hexose monophosphate and glucose.

---

[2] The hexose monophosphate obtained by this method is, according to Smythe, a mixture of about 22 per cent fructose-6-phosphate, 60 per cent glucose-6-phosphate, and 18 per cent mannose-6-phosphate.

Phosphorylation of Hexose Monophosphate and Glucose

The phosphorylation of adenylic acid and creatine is only slight owing to the presence in the brain dispersions of a very active adenosine triphosphatase. However, in the presence of either hexose monophosphate or glucose, small amounts of adenylic

TABLE I

Phosphorylation of Adenylic Acid and Creatine

Enzyme dialyzed 6 hours. All samples contained 0.1 mg. of Mg^{++}, 0 004 M fumarate, 0 014 M pyruvate, 0.021 M phosphate (Experiments 1 and 2), or 0.017 M phosphate (Experiment 3); Experiments 2 and 3 also with 0 00012 M adenylic acid (0.009 mg. of P). Incubated in air at 28°.

Experiment No.	P acceptor	NaF	Incubation time	P				
				True inorganic	Determined directly*	Phospho-creatine	7 min. hydrolysis in 1 0 N HCl at 100°	Pyrophosphate
		$M \times 10^{-3}$	$M \times 10^{-3}$	min	mg.	mg.	mg.	mg.	mg.
1	Adenylic acid (4 5)†	50	0		1.66		1.66	0 00
			10		1.57		1.62	0.05
			20		1.61		1 66	0 05
			30		1.63		1 67	0 04
2	Creatine (14 0)‡	50	0	1.66	1.65	0.00		
			10	1.57	1.64	0.07		
			20	1.60	1.70	0.10		
			30	1 64	1.68	0.04		
3§	Creatine (14.0)	0	0	1.31	1.32	0.01		
		0	20	1.29	1.32	0.03		
		40	20	1.28	1.36	0.08		

* Inorganic P in the absence of phosphocreatine.

† 0.35 mg. of P.

‡ Equivalent to 1.1 mg of P.

§ In this experiment the samples contained also 0.04 M Na_2CO_3-$NaHCO_3$ mixed in the proportion of 1:4.

acid, the remaining components of the pyruvate oxidation system, and sodium fluoride, there is a rapid disappearance of inorganic phosphate in relation to the oxygen uptake of the preparations[3]

[3] The inorganic P disappearing is given as esterified P in the tables. Net oxygen uptakes are always given; *i e*, the difference between the oxygen uptake of complete samples minus that of samples without pyruvate + fumarate The latter was mostly insignificant.

(Tables II and V). In the absence of pyruvate + fumarate, and with brain dispersions which have been dialyzed for a sufficiently long time (4 or more hours), there is neither oxygen consumption nor phosphorylation. For a given brain preparation and a given time of incubation, the ratio of atoms of phosphorus esterified to molecules of oxygen taken up is nearly constant (Tables II and V, last column) whether the P acceptor be hexose monophosphate or glucose, in spite of the different magnitude of the oxygen uptake in each case.

The disappearing inorganic phosphate is esterified with either hexose monophosphate or glucose to hexose diphosphate which, by the action of the zymohexase (13) present in the brain prepara-

TABLE II

Phosphorylation of Hexose Monophosphate and Glucose

Enzyme dialyzed 4 hours; incubated 35 minutes in air at 38°. All samples contained 0.2 mg. of Mg^{++}, 0 023 M phosphate, 0.0013 M adenylic acid (0 087 mg of P), and 0 02 M NaF. O$_2$ uptake and esterification of phosphate were measured during the last 30 minutes.

Sample No.	Hexose monophosphate	Glucose	Fumarate	Pyruvate	O$_2$ uptake	P esterified	$\dfrac{\text{Atoms P}}{\text{Moles O}_2}$
	M × 10⁻³	M × 10⁻³	M × 10⁻³	M × 10⁻³	c mm.	mg	
1	14*				0	0 00	0.0
2		25			0	0 00	0.0
3	14		4	12	237	0 55	1.7
4		25	4	12	317	0 85	1 9
5		25	4	12	323	0 86	1 9

* 0.96 mg. of P.

tions, is partly converted into alkali-labile triose phosphate (Table III). Demonstration that the product of esterification is hexose diphosphate has been achieved in three ways: (a) by determining the hydrolysis curves according to Lohmann (in 1.0 N HCl at 100°) of the esters present in the trichloroacetic acid centrifugates at the end of the experiment; (b) by heating some samples, prior to deproteinization, for 5 minutes at 60° after the usual incubation of 35 minutes at 38°; and (c) by direct estimations of fructose.

Hydrolysis Curves—From the data given in Table III, the percentage hydrolysis of the total ester P with exclusion of triose phosphate (total acid-soluble minus alkaline P), after 7, 30, 60,

and 180 minutes hydrolysis, respectively, was calculated. Hydrolysis of the triose phosphate present was corrected for on the basis of a hydrolysis curve published by Kiessling (14). In this way the curves of Fig. 1 were obtained. The small amounts of adenylic acid added to each sample and the eventual presence of traces of some phosphoric ester in the brain dispersions are a source of some error in the above calculation. From Table III it can be calculated that $1.64 - 0.77 = 0.87$ mg. of inorganic P has been esterified in 35 minutes in the experiment with hexose monophosphate (Sample 3). This would make $0.87 \times 2 = 1.74$ mg. of hexose diphosphate (including triose phosphate) P, whereas $2.75 - 0.77 = 1.98$ mg. represents the total ester P present.

TABLE III

P Fractions of Experiments of Table II at End of 35 Minutes Incubation

Sample No	P						
	Determined directly	Alkaline treatment*	Hydrolysis in 1.0 N HCl at 100°				Total acid-soluble
			7 min.	30 min.	60 min.	180 min.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg*
1	1.64	1 66	1.71	1 80	1.86	2.00	2.70
2	1.58	1 58	1.59	1.59	1 59	1 62	1 71
3	0.77	1 14	1.45	2 01	2 21	2.54	2.75
4	0.25	0.58	0 79	1.17	1 31	1 55	1.69
5	0.24	0.58	0 75	1.20	1.33	1.58	1 73

* Determined after treatment with 1.0 N NaOH for 20 minutes at room temperature (inorganic + triose phosphate P).

Thus $1.98 - 1.74 = 0.24$ mg. of P (or 12 per cent of the total) is ester which has not arisen by phosphorylation of hexose monophosphate. Conditions are similar in the experiments with glucose. For this residue, however, no correction can be applied; it is even uncertain how much of it is adenylic acid, as this may have been broken down to a greater or lesser extent by the specific phosphatase found by Reis (15) in brain and nerve tissue. The curves obtained as outlined above agree with that of fructose-1,6-diphosphate (Fig. 1).

Further evidence for hexose diphosphate formation is the increase in triose phosphate when the brain samples, after 35 minutes incubation at 38°, are heated at 60° for 5 minutes prior to deproteinization (Table IV). This indicates that the triose phosphate is formed from hexose diphosphate by the zymohexase
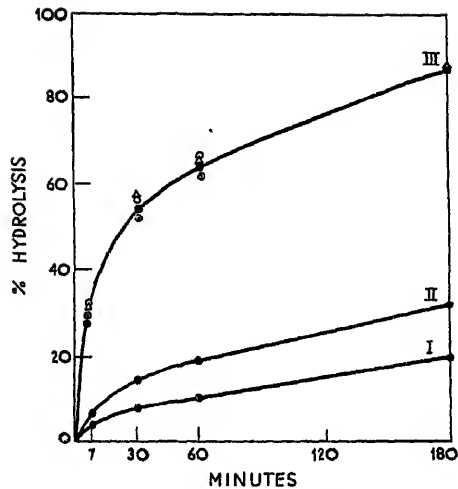
FIG. 1. Hydrolysis curves. Curve I, hexose-6-phosphate in aqueous solution. Curve II, trichloroacetic acid filtrate after aerobic incubation of hexose-6-phosphate with dialyzed brain enzyme (Table III, Sample 1). Curve III, ● fructose-1,6-diphosphate in aqueous solution, ○ trichloroacetic acid filtrate after aerobic incubation of dialyzed brain enzyme with fumarate, pyruvate, and hexose-6-phosphate as P acceptor (Table III, Sample 3); △ same but glucose as P acceptor (Table III, Samples 4 and 5); ⊙ trichloroacetic acid filtrate after anaerobic incubation of dialyzed brain enzyme with phosphoglycerate and glucose (Table XI, Samples 4 and 10).

TABLE IV

Phosphorylation of Glucose; Formation of Triose Phosphate and Fructose Diphosphate

Enzyme dialyzed 4 hours. All samples contained 0.2 mg. of Mg^{++}, 0.023 M phosphate, 0.0013 M adenylic acid (0 087 mg. of P), 0.004 M fumarate, 0.012 M pyruvate, 0.025 M glucose, and 0.02 M NaF.   Gas, air.

Sample	O_2 up-take*	P				Hexose diphosphate	
		Deter-mined directly	Alka-line treat-ment	Triose phos-phate	Esteri-fied	From P esti-mation	From fructose esti-mation
	c.mm.	mg	mg.	mg	mg	mg.	mg
Initial		1.63	1.63	0.00			0 00
Incubated 35 min., 38°	294	0 35	0.64	0 29	1 28	0.99	1.05
" 35 " 38° + 5 min , 60°	282	0 51	0 93	0.42	1.12	0.70	0.77

* Measured for the period of 5 to 35 minutes.

(aldolase) reaction (13).   Table IV shows further that the values for hexose diphosphate P, as obtained by direct determination of fructose, agree with those calculated on the assumption that all of the esterified phosphate, with the exception of that appearing as triose phosphate, is hexose diphosphate P.

In brain dispersions, hexose diphosphate and triose phosphate account for all of the esterified phosphate.   In some experiments, aliquots of filtrates from samples incubated with and without hexose monophosphate as P acceptor were treated with lead ace-

<div align="center">

TABLE V

Time Course of Esterification

</div>

Enzyme dialyzed in Experiment 1, 2.5 hours; in Experiment 2, 5 hours. All samples contained 0.2 mg of Mg^{++}, 0 022 M phosphate, 0.0013 M adenylic acid (0.087 mg. of P), 0 004 M fumarate, 0.012 M pyruvate, and 0 02 M NaF Incubated in air at 38°.

Experiment No.	P acceptor	Incubation time	O_2 uptake		P esterified		Pyruvic acid removed		Atoms P / Moles O_2
	$M \times 10^{-3}$	*min.*	*c mm.*	*micromoles*	*mg.*	*microatoms*	*mg.*	*micromoles*	
1	Hexose mono-	5-10	32	1.43	0 18	5 81			4.05
	phosphate	5-15	85	3.80	0 40	12 95			3.41
	(14.0)*	5-25	149	6.65	0 61	19 60			2 95
		5-35	181	8.10	0.70	22.60			2 78
2	Glucose (25.0)	5-10	80	3.56	0 46	14.80	0 29	3.30	4.15
		5-15	150	6.70	0 70	22 60	0.56	6 38	3.37
		5-25	245	10.90	0 95	30.70	0 71	8 10	2 82
		5-35	295	13.20	1 02	33 00	0.86	9.80	2.50

* 0.96 mg. of P.

tate and the precipitate worked up for phosphoglyceric acid estimation by the method of Rapoport (16), but none could be detected.   Hence the phosphorylation here measured cannot be due to any extent to triose phosphate oxidation.

Time Course of Esterification—With either hexose monophosphate or glucose as P acceptor, 4 atoms of phosphorus are esterified for each molecule of oxygen consumed during the second 5 minutes of incubation (Table V).   This ratio gradually decreases with time and, for the period 5 to 35 minutes, it usually has a value around 2.   The decrease with time of the esterification rate was

observed by Kalckar with kidney extracts (6); he also found that esterification of inorganic phosphate with a P acceptor was depressed by addition of the phosphorylation product (*i.e.* phosphorylation of glucose was inhibited by hexose diphosphate), a fact which has been here confirmed (Table VI). With the brain dispersions the oxygen consumption of the complete pyruvate oxidation system plus P acceptor is always somewhat depressed in the presence of hexose diphosphate, but inhibition of the esterification is relatively much larger, as reflected by the lower $P:O_2$ ratios obtained.

TABLE VI

Inhibition of Phosphorylation by Hexose Diphosphate

Enzyme dialyzed 4 hours; same additions as in Table V. Incubated 35 minutes in air at 38°. O_2 uptake measured for the last 30 minutes, P esterification measured for the whole period of incubation.

P acceptor	Hexose diphosphate	O_2 uptake	P esterified	$\dfrac{\text{Atoms P}}{\text{Moles } O_2}$
$M \times 10^{-3}$	$M \times 10^{-3}$	*c.mm.*	*mg.*	
Hexose monophosphate (17 0)*	0	139	0 60	3.1
	7†	108	0.21	1 4
Glucose (23 0)	0	256	0 95	2 7
	7	166	0 36	1.6

* 1.3 mg of P
† 1.1 mg of P.

The high $P:O_2$ values at the beginning might be due in part to a phosphorylation connected with some anaerobic reaction, mainly occurring during the first few minutes of incubation. This possibility, however, was excluded; when the samples are incubated in nitrogen, under otherwise identical conditions as in the aerobic experiments of Table II, there is neither removal of pyruvate nor phosphorylation.

The initial rate of phosphorylation is extremely high. Thus in Experiment 2 of Table V, with glucose as P acceptor, 0.46 mg. of P were esterified at 38°, during the second 5 minutes, by an amount of enzyme corresponding to approximately 300 mg. of fresh brain, or about 18 mg. of P per gm. of tissue per hour.

Cocarboxylase and Esterification—Dependence of phosphorylation on the oxidation of pyruvic acid is also observed if the con-

centration of cocarboxylase be a limiting factor in the pyruvate oxidation system, as is the case when brain dispersions from pigeons with acute beriberi are used (2, 4). This is shown in Table VII. With no cocarboxylase added, oxygen uptake and pyruvate removal are lower; phosphorylation is proportionally depressed. Addition of cocarboxylase produces increased phosphorylation in relation to the higher oxygen consumption and to the increased removal of pyruvate; the $P:O_2$ ratios remain unchanged. Experiment 3 (Table VII) is particularly demonstrative, since the activity of the system, without added cocarboxylase, was lower

TABLE VII

Effect of Cocarboxylase on Esterification

Brain enzyme from vitamin B_1-deficient pigeons, dialyzed 5 hours. Additions as in Table V. Incubated 35 minutes in air at 38°. O_2 uptake, P esterification, and pyruvate removal measured during the last 30 minutes.

Experiment No	P acceptor	Cocarboxylase added	O_2 uptake	P esterified	Pyruvic acid removed	$\dfrac{\text{Atoms P}}{\text{Moles } O_2}$
	$\mu \times 10^{-3}$	γ	c.mm.	mg.	mg.	
1	Hexose mono-phosphate (14 0)*	0	115	0 33	0 42	2 07
		‸ 2†	191	0 53	0 86	2.00
2	" " (14 0)	0	137	0.38	0.52	1 99
		2	212	0 65	0.94	2.22
3	Glucose (25.0)	0	66	0 25		2.73
		2	188	0.76		2.92

* 0 96 mg. of P.
† Actually 3 γ of cocarboxylase of 65 per cent purity.

than is usually obtained; its addition trebled both oxygen uptake and phosphorylation.

Adenylic Acid and Esterification—In brain dispersions which have been dialyzed sufficiently long to remove as much as possible of the preformed "adenine nucleotide" without causing irreversible inactivation of the enzymes, esterification of inorganic phosphate with hexose monophosphate or glucose, in the presence of sodium fluoride and all the components of the pyruvate oxidation system except adenylic acid, is negligible (Table VIII). The effect of adenylic acid is catalytic; addition of 1 mg., with 0.087 mg. of P, causes (*i.e.* in Experiment 2, Table VIII) the esterification of 0.96

$- 0.10 = 0.86$ mg. of P, or 10 times as much as the adenylic acid
P added, not including the phosphate esterified during the first
5 minutes of temperature equilibration. Without adenylic acid
the oxygen uptake is very low (4), but lack of the nucleotide
affects phosphorylation even more and low $P:O_2$ ratios result.
In Experiment 1 of Table VIII pyruvic acid was determined as
described in a previous section. On addition of adenylic acid the
oxygen consumption is increased to a greater extent than is the
removal of pyruvic acid, the oxygen to pyruvate ratio being

TABLE VIII

Necessity of Adenylic Acid for Esterification

Enzyme dialyzed 7 hours. All samples contained 0 2 mg. of Mg^{++},
0.022 M phosphate, 0.004 M fumarate, 0.012 M pyruvate, and 0.02 M NaF.
Incubated 35 minutes at 38°. O_2 uptake, P esterification, and pyruvic acid
disappearance measured during the last 30 minutes.

Experiment No.	P acceptor	Adenylic acid added	O_2 uptake	P esterified	Pyruvic acid removed	$\dfrac{\text{Atoms P}}{\text{Moles } O_2}$	$\dfrac{\text{Moles } O_2}{\text{Moles pyruvic acid}}$
		$M \times 10^{-3}$	$M \times 10^{-3}$	c.mm.	mg.	mg.		
1	Hexose mono- phosphate (14.0)*	0 0	84	0.15	0.56	1.29	0.59
		1 3†	217	0 68	0 83	2.27	1.03
2	Glucose (25.0)	0.0	121	0.10		0.60	
		1.3	302	0 96		2.30	

* 0.96 mg. of P.

† 0.087 mg. of P.

doubled. This confirms previous observations, in which pyruvate
had been determined by a different method (4), and the conclusion
then reached that adenylic acid causes not only an increased
removal but also a more complete oxidation of pyruvic acid.

*Phosphate Transfer from Phosphopyruvic and Phosphoglyceric
Acids to Hexose Monophosphate and Glucose*—Kalckar (6, 7) ob-
served that respiring suspensions of minced kidney cortex would
esterify inorganic phosphate with adenylic acid to adenosine
polyphosphate, but failed to obtain transfer of phosphate from
adenosine triphosphate to glucose. Similarly Colowick *et al.*

(8), although noticing the improved respiration caused by addition of adenylic acid to autolyzed kidney extracts and its necessity for aerobic glucose phosphorylation, were also unable to obtain transfer of phosphate from adenosine triphosphate to glucose.[4] From these results it would appear that aerobic phosphorylation of glucose would not take place by preliminary phosphorylation of adenylic acid to adenosine polyphosphate and transfer of phosphate from the latter to the sugar. The experiments to be reported here, however, show that, under different experimental conditions, such a transfer can be demonstrated to occur in brain dispersions.

In preparations with a high adenosine triphosphatase activity little success is to be expected when relatively large amounts of adenosine triphosphate are used as P donator. In the present experiments therefore either phosphopyruvic or phosphoglyceric acid was used as (indirect) P donator, and catalytic amounts of adenylic acid as carrier (hence small amounts at a time of adenosine polyphosphate as direct donator) of phosphate to either hexose monophosphate or glucose; as previously reported (1), phosphorylation to hexose diphosphate of either P acceptor is catalyzed by the brain dispersions under these conditions. In the absence of adenylic acid there is of course no reaction. The experiments were carried out under strictly anaerobic conditions[5] to prevent an eventual esterification through oxidation of some of the pyruvic acid arising by dephosphorylation of phosphopyruvate, and, further, in the presence of iodoacetate to prevent dismutation between pyruvate and triose phosphate, a reaction which is coupled with esterification (18-20).

In the experiment of Table IX, with 0.02 M sodium fluoride, the whole of the added phosphopyruvate (with 0.49 mg. of P) was broken down in 15 minutes at 38°. Half of its P (0.25 mg.) appeared as inorganic phosphate; the rest as an ester of which 0.15 mg. was triose phosphate. Phosphopyruvate was determined as the difference between the phosphate of an aliquot of

---

[4] Transfer of phosphate from adenosine triphosphate to glucose with brain extracts was recently reported by Geiger (17). Colowick, Kalckar, and Cori have now found it to occur in heart extracts (*J. Biol Chem.*, **137**, 343 (1941)).

[5] In nitrogen purified by passage through copper at 600°.

filtrate treated with 0.03 N iodine in 1.0 N sodium hydroxide for 20 minutes at room temperature (I_2 value) and that of another treated only with 1.0 N sodium hydroxide under the same conditions (alkaline value). The .difference between ashing and I_2 phosphate values is ester phosphate other than triose phosphate or phosphopyruvate. In Table IX this difference was 0.65 mg. of P before incubation, corresponding to the added hexose monophosphate; after incubation it was 0.76 mg. of P. Thus 0.76 − 0.65 = 0.11 mg. of P is newly formed ester other than triose

TABLE IX

Transfer of Phosphate from Phosphopyruvate to Hexose Monophosphate

Enzyme dialyzed 6.5 hours. All samples contained 0.1 mg. of Mg^{++}, 0.01 M phosphate, 0.009 M hexose monophosphate (0 65 mg of P), 0 007 M phosphopyruvate (0.5 mg. of P), 0.001 M iodoacetic acid, and 0.02 M NaF Incubated 15 minutes in nitrogen at 38°.

Sample	P			
	Determined directly	Triose phosphate	Phospho-pyruvate	Remaining ester†
	mg.	*mg.*	*mg.*	*mg.*
Initial	0 72	0.00	0.49	0.65
Incubated without adenylic acid	0.76	0.00	0 47	0 62
" with 0.0012 M adenylic acid*. .	0.97	0.15	0.00	0 76
Change on incubation with adenylic acid . ..	+0.25	+0.15	−0.49	+0 11

* 0.087 mg. of P.

† Total acid-soluble minus I_2 values of P.

phosphate. In experiments with glucose as P acceptor this ester has been identified as hexose diphosphate.

Table X gives experiments with phosphoglycerate. Here of course no fluoride can be used. When no adenylic acid was added, there was practically no reaction except for the appearance of phosphopyruvate with an approximately equivalent disappearance of phosphoglycerate[6] owing to the establishment of the equilibria, 3-phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate $\rightleftharpoons$ phosphopyruvate. With adenylic acid, the whole of the phospho-

[6] Phosphoglycerate P was determined as the difference between ashing and 180 minutes hydrolysis in 1.0 N HCl at 100°.

glycerate was broken down irrespective of the absence or presence of hexose monophosphate or glucose, but whereas in the former case all the phosphoglycerate P is set free as inorganic phosphate, in the latter 25 to 30 per cent of. it failed to appear as inorganic phosphate and was recovered as triose phosphate and hexose

TABLE X

Transfer of Phosphate from Phosphoglycerate to Hexose Monophosphate and Glucose

Enzyme dialyzed 4.5 hours. All samples contained 0.2 mg. of Mg^{++}, 0.009 M phosphate, and 0.001 M iodoacetic acid. Incubated in nitrogen.

Sample No	P acceptor	Phospho-glyce-rate added	Incuba-tion period	Ad-enylic acid added	P				
					Deter-mined directly	Triose phos-phate	Phos-phopy-ruvate	Phos-glycer-ate	Hex-ose di-phos-phate*
	$M \times 10^{-3}$	$M \times 10^{-3}$	min.	$M \times 10^{-3}$	mg.	mg.	mg.	mg.	mg.
1	None	20†	Initial	0 0	0.65	0 00	0.02	1 54	
2	Glucose (24)	20	20 (38°)	0 0	0.71	0 02	0.38	1.13	
3	None	20	20 (38°)	1.2‡	2 28	0 00	0.00	0.00	
4	Glucose (24)	20	20 (38°)	1 2	1.74	0.17	0.00	0.00	
5	Hexose mon-ophosphate (18)§	20	20 (38°)	1.2	1.90	0.23	0.00		
6	None	23‖	Initial	0 0	0 68	0 00	0.07	1 76	0.00
7	Glucose (23)	23	20 (38°)	0 0	0 75	0.00	0.54	1.35	0.00
8	None	23	20 (38°)	1.2	2.56	0.00	0.00	0.00	0.00
9	Glucose (23)	23	20 (38°)	1.2	2 03	0 16	0.00	0.00	0.44
10	" (23)	23	20 (38°)	1.2	2 08	0.18	0.00	0.00	
11	" (23)	23	20 (38°) +5 (60°)	1 2	2.08	0 27			

* Determined as fructose.
† 1.5 mg. of P.
‡ 0.087 mg. of P.
§ 1 3 mg. of P.
‖ 1.7 mg. of P.

diphosphate. The hydrolysis curve of the residual ester was determined, in samples with glucose as P acceptor, as previously described. This has been done with Samples 4 and 10 (Table X); the analytical data are given in Table XI. The curve agrees with that of fructose-1,6-diphosphate (Fig. 1). Table X shows further

that incubation for 5 minutes at 60° at the end of the experiment markedly increases the amount of triose phosphate (*cf.* Samples 10 and 11). Direct estimation of fructose also indicates that the residual ester is hexose diphosphate. Thus in Sample 9, 2.56 − 2.03 = 0.53 mg. of P from phosphoglycerate are bound in ester form, of which 0.16 mg. of P is triose phosphate; the remainder, 0.37 mg. of P, agrees with the hexose diphosphate P (0.44 mg.) obtained by the fructose estimation.

The quantitative relationships in the above experiments require some consideration. In the presence of fluoride half of the phosphopyruvate P is set free as inorganic phosphate, and the remaining half is tranferred to the P acceptor (Table IX); without fluoride more phosphate appears as inorganic and only 25 to 30

TABLE XI

P Fractions of Experiments of Table X at End of Incubation

Sample No.	P					
	Directly determined	Hydrolysis in 1 0 N HCl at 100°				Total acid-soluble
		7 min	30 min.	60 min.	180 min.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
3	2 28				2 28	2 32
4	1.74	1 94	2.05	2.13	2 18	2.27
8	2.56				2 63	2.67
10	2.08	2.29	2 42	2 47	2.60	2.65

per cent is transferred to the P acceptor (Table X). Without fluoride, however, aerobic phosphorylation is also much decreased (*cf.* the next section). In anaerobic transfer experiments with phosphopyruvate, hexose monophosphate, and fluoride, reduction to 3 minutes of the incubation time resulted in the same relations; *i.e.* half of the phosphopyruvate phosphate was set free and half tranferred to hexose monophosphate.

This is the same relation which was found by Ostern *et al.* (21) for the transfer of phosphate from adenosine triphosphate to hexose monophosphate in autolyzed muscle extracts. The reactions in brain dispersions in the presence of fluoride can be formulated as follows:

(1)   4 phosphopyruvic acid + 2 adenylic acid = 2 adenosine triphosphate + 4 pyruvic acid

(2) 2 adenosine triphosphate + 2 hexose monophosphate + $2H_2O$
$\quad$ = 2 hexose diphosphate + $2H_3PO_4$ + 2 adenylic acid
or
(3) 2 adenosine triphosphate + 1 glucose + $2H_2O$ = 1 hexose diphosphate
$\quad\quad$ + $2H_3PO_4$ + 2 adenylic acid
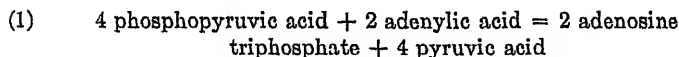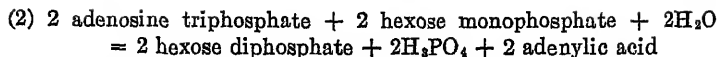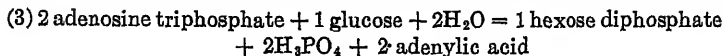
Reaction (2) is that of Ostern *et al.* The sum of Equations 1 and 2 is the over-all reaction for the phosphorylation of hexose monophosphate:

(4) $\quad$ 4 phosphopyruvic acid + 2 hexose monophosphate + $2H_2O$
$\quad\quad$ = 2 hexose diphosphate + $2H_3PO_4$ + 4 pyruvic acid
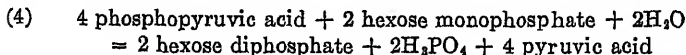
Although phosphorylation of hexose monophosphate by transfer of phosphate from adenosine triphosphate was known to occur both in yeast and muscle, phosphorylation of glucose by the same mechanism appeared to be limited to the former. Muscle can only bring about the reaction in the presence of hexokinase, an enzyme of the nature of a heterophosphatese (22). However, other tissues which glycolyze glucose, such as brain and kidney, contain hexokinase (23) and might be expected to catalyze the phosphorylation of glucose by adenosine triphosphate and transfer of phosphate from phosphopyruvate.

Effect of Poisons on Aerobic Phosphorylation—The effect of fluoride has already been mentioned. Relative to the oxygen consumption phosphorylation is much decreased in the absence of fluoride. This may not be true in absolute terms, owing to the depression of respiration by fluoride which (with 0.04 M NaF) may be reduced to 50 per cent of its normal value (Table XII, Experiment 1). In the absence of fluoride the $P:O_2$ ratio is some 30 to 40 per cent of that in its presence. Kalckar (6) emphasized the necessity of fluoride for the study of aerobic phosphorylations in kidney, and ascribed its effect to inhibition of acid phosphatases which would split the phosphorylation product. Colowick *et al.* (8) also noticed the effect of fluoride but could not observe inhibition of phosphatase action by the poison. The only well known specific effect of fluoride, namely inhibition of the equilibrium reaction 2-phosphoglycerate $\rightleftharpoons$ phosphopyruvate, cannot explain its action on the aerobic phosphorylation in brain, under the conditions of the present experiments, where no phosphoglycerate is formed from triosephosphate. As the experiments with brain, and probably also those with kidney preparations, are car-

ried out in the presence of a strong adenosine triphosphatase, a depression by fluoride of the activity of this enzyme might well explain its effect.

Iodoacetic acid (0.001 M) produces some inhibition of the respiration of the brain pyruvate oxidation system, but decreases phosphorylation only to the same extent as respiration, leaving the P:O_2 values practically unchanged (Table XII). On the other hand iodoacetic acid blocks, as is well known, the esterification of inorganic phosphate connected with dehydrogenation of

TABLE XII

Effect of Fluoride and Iodoacetate on O_2 Uptake and Phosphorylation

Enzyme dialyzed 4 to 6 hours. All samples contained 0.2 mg of Mg^{++}, 0.022 M phosphate, 0 0013 M adenylic acid (0 087 mg. of P), 0 004 M fumarate, 0.012 M pyruvate, and either 0.009 M hexose monophosphate (0.63 mg. of P) or 0.025 M glucose   Incubated 35 minutes in air at 38° O_2 uptake and P esterification measured during the last 30 minutes.

Experiment No.	P acceptor	NaF	Iodoacetic acid	O_2 uptake	P esterified	Atoms P / Moles O_2
		$M \times 10^{-3}$	$M \times 10^{-3}$	c.mm.	mg.	
1	Hexose mono-	0	0	420	0.31	0.55
	phosphate	40	0	210	0.39	1.30
2	Glucose	20	0	284	0 79	2.02
		0	1	225	0 21	0 68
		20	1	222	0.53	1 73
3	"	0	0	192	0.12	0.45
		20	1	128	0.31	1 76

triose phosphate by inhibiting the triose phosphate dehydrogenase (18, 24).

The effect of arsenite was also investigated. Krebs and Johnson (25) found arsenite to be a specific inhibitor of the dehydrogenation of α-keto acids. The fumarate-catalyzed oxidation of pyruvate in brain dispersions is completely blocked by arsenite and so is the accompanying phosphorylation (*cf.* Table XIV). That inhibition of phosphorylation is secondary to that of pyruvate oxidation is proved by the fact that 0.008 M sodium arsenite has no effect whatever on the phosphorylation of glucose by transfer of phosphate from phosphoglyceric acid. Thus in Table XIII,

2.05 − 1.72 = 0.33 mg. of P was transferred to glucose whether arsenite was present or not.

Succinate Oxidation and Phosphorylation—Colowick *et al.* (8) found that aerobic phosphorylation of glucose in kidney extracts was obtained by oxidation of various dicarboxylic acids, among them succinic acid, and expressed the view that oxidation of any substrate involving Szent-Györgyi's fumaric acid catalysis would be an indirect source of phosphorylation. They also reported that oxidation of pyruvic acid catalyzed by fumarate produced phosphorylation of glucose.

TABLE XIII

Effect of Arsenite on Transfer of Phosphate from Phosphoglycerate to Glucose

Enzyme dialyzed 4 hours. All samples contained 0 2 mg. of Mg^{++}, 0.008 M phosphate, 0.0011 M adenylic acid (0 087 mg. of P), 0.018 M phosphoglycerate (1.4 mg. of P), and 0.001 M iodoacetic acid. Incubated 10 minutes in nitrogen at 38°.

| Sample | P acceptor | Arsenite | P | | |
			Directly determined	Phosphoglycerate split	Transferred
	$M \times 10^{-4}$	$M \times 10^{-4}$	*mg.*	*mg.*	*mg.*
Initial	None	0	0 65		
Incubated	"	0	2.05	1.40	0 00
"	Glucose (22)	0	1.72	1 40	0.33
"	None	8*	2.02	1.37	0.00
"	Glucose (22)	8	1.70	1.37	0.32

* 2 mg. of As_2O_3.

If succinate oxidation were the only source of phosphorylation in the oxidation of pyruvate catalyzed by C_4 dicarboxylic acids, one would expect the $P:O_2$ ratio to be the same or higher with succinate than with pyruvate-fumarate; this is not the case, the ratio being lower with succinate (Table XIV). When succinate is oxidized by brain dispersions, owing to the presence of fumarase and malic dehydrogenase, pyruvate is always formed from oxalacetate and is undoubtedly oxidized to some extent. This can be avoided with arsenite. Table XIV shows that in the presence of arsenite the $P:O_2$ ratio of succinate oxidation drops to a half or

less of the value obtained (without arsenite) when pyruvate is oxidized.

Experiment No.	P acceptor	Fumarate	Pyruvate	Succinate	Arsenite	O_2 uptake	P esterified	Atoms P / Moles O_2
		$M\times10^{-2}$	$M\times10^{-3}$	$M\times10^{-2}$	$M\times10^{-3}$	c.mm.	mg.	
1	Hexose monophosphate	4	12	0	0.0	178	0 68	2.76
		4	12	0	0 8*	0	0 12	
		0	0	30	0.0	158	0 37	1 69
		0	0	30	0.8	135	0.24	1.28
2	Glucose	4	12	0	0.0	320	1 20	2.72
		4	12	0	8.0	19	0.00	0 00
		0	0	30	0.0	276	0 77	2 02
		0	0	30	8.0	178	0 23	0.94

* 0.2 mg. of As_2O_3.

DISCUSSION

Esterification of inorganic phosphate with a P acceptor is connected with two of the major dehydrogenations in carbohydrate breakdown, namely dehydrogenation of triose phosphate (18, 19, 26, 27) and pyruvate. The mechanism of the "coupling" between triose phosphate dehydrogenation and esterification has been clarified by the work of Warburg and his associates (28, 29). Glyceraldehyde phosphate takes up inorganic phosphate (whether by enzymatic action or otherwise is still unknown) and is converted into glyceraldehyde diphosphate which is then dehydrogenated to 1,3-diphosphoglyceric acid; this phosphorylates adenylic acid (or adenosine diphosphate) and passes into the more stable 3-phosphoglyceric acid.

The connection between esterification and oxidation of pyruvic acid here studied would seem to be due to a similar reaction mechanism. Such a mechanism has been postulated by Lipmann

(30) for the simpler oxidative decarboxylation of pyruvic acid to acetic acid and carbon dioxide by preparations of lactic acid bacteria, supported by the fact that the hypothetic phosphorylated dehydrogenation product, acetyl phosphate, transfers its phosphate to adenylic acid in the presence of the bacterial enzymes. More recently (31) he has found that a (acid and alkali) labile phosphorylated compound is formed during dehydrogenation of pyruvate by the bacterial system.

The pyruvate oxidation system of animal tissues is undoubtedly more complicated, since oxidation of pyruvate is more complete (32) and requires the cooperation of C_4 dicarboxylic acids, which also seem to take a part in phosphorylation. Here acetyl phosphate does not appear to be an intermediate (33). On the other hand, the necessity of "adenine nucleotide" for the oxidation is a point of analogy with the triose phosphate oxidation system which is not shared by Lipmann's bacterial preparations. The mechanism of phosphorylation in brain dispersions can be pictured as follows:

With catalytic amounts of adenylic acid, and in the absence of further P acceptors, the adenosine triphosphate, formed by reaction of adenylic acid with the hypothetic phosphorylated oxidation product of pyruvic acid, is rapidly dephosphorylated by the adenosine triphosphatase, and adenylic acid is regenerated for further reaction; the concentration of inorganic phosphate remains unchanged. It is due to the presence of adenosine triphosphatase that oxygen consumption can take place independently of the phosphorylation of a P acceptor, but, in the absence of added adenylic acid, the concentration of preformed "adenine nucleotide" in the dialyzed dispersions is too low to keep the above mechanism working at a sufficiently high rate, and the rate of oxygen uptake falls below normal. In the presence of both adenylic acid (in catalytic amounts) and phosphate acceptors for the labile P groups of adenosine triphosphate, such as hexose monophosphate or glucose, a greater or lesser proportion of the adenosine triphosphate escapes the action of adenosine triphosphatase by transferring phosphate to the acceptor with formation of hexose diphosphate; an equivalent amount of inorganic phosphate disappears The fact that phosphorylation of adenylic acid is an obligate step in dehydrogenation of both triose phosphate and

pyruvate makes it the main mechanism by which the energy of respiration is made available to the tissues.

The part taken by the catalytic action of C_4 dicarboxylic acids in phosphorylation is obscure.· The fact that, at a certain time, there are at least 4 atoms of phosphorus esterified to 1 molecule of oxygen consumed would seem to support the view (1) that there must be more than one point of esterification in the chain of reactions leading to the transport of one hydrogen pair from pyruvate to their ultimate acceptor, oxygen.[7]

The phosphorylation linked with oxidation of pyruvate, relatively resistant to iodoacetic acid, throws light on early observations upon aerobic resynthesis of creatine phosphate in iodoacetic acid-treated muscles (35), and on more recent observations of Belitzer (36) upon phosphorylation of creatine in minced muscle oxidizing lactate in the presence of bromoacetate.

SUMMARY

1. Dialyzed dispersions from pigeon brain, in the presence of sodium fluoride and all the components of the pyruvate oxidation system, show a limited capacity to phosphorylate adenylic acid or creatine.

2. With catalytic amounts of adenylic acid, added hexose monophosphate or glucose is phosphorylated to hexose diphosphate with uptake of an equivalent amount of inorganic phosphate. The presence of adenylic acid is indispensable.

3. Under the conditions of these experiments phosphorylation is connected with the oxidation of pyruvate catalyzed by C_4 dicarboxylic acids, since (a) without pyruvate-fumarate there is neither oxygen uptake nor phosphorylation; (b) in brain dispersions from vitamin B_1-deficient pigeons esterification is increased by addition of cocarboxylase to the same extent as removal of pyruvate and

---

[7] After completion of the manuscript, a paper by Belitzer and Tsibakowa (34) came to my knowledge, in which phosphorylation of creatine, in minced muscle and heart, was found to be linked with oxidation of various substrates including pyruvate; also of succinate (in the presence of arsenite) with a lower $P:O_2$ ratio, as was here found With preformed substrates, $P:O_2$ ratios of 4 or higher were found The authors put forward the view, here expressed, that intermediate reactions in the hydrogen transfer must also be connected with phosphorylation.

oxygen consumption; (c) arsenite blocks the esterification by inhibiting the oxidation of pyruvate.

4. Under certain conditions the ratio of atoms of phosphorus esterified to molecules of oxygen·taken up is constant. This ratio has a maximum value of 4 at the beginning, but decreases with time owing to inhibition of esterification by the accumulating phosphorylation product.

5. With catalytic amounts of adenylic acid, brain dispersions catalyze phosphorylation to hexose diphosphate, of either hexose monophosphate or glucose, by anaerobic transfer of phosphate from phosphopyruvic or phosphoglyceric acid, showing that the labile phosphate groups of adenosine triphosphate are transferred to either of the two P acceptors. Thus the necessity of adenylic acid for aerobic phosphorylation can be best interpreted by assuming that oxidation of pyruvate is linked with phosphorylation of adenylic acid to adenosine polyphosphate which then transfers its labile phosphate to hexose monophosphate or glucose.

6. Relative to the oxygen consumption phosphorylation is much decreased in the absence of fluoride. The effect of fluoride is believed to be due to a depression of the activity of adenosine triphosphatase. Both oxygen uptake and phosphorylation are relatively resistant to iodoacetic acid and are depressed by it to the same extent.

7. Oxidation of succinate by brain dispersions is also accompanied by phosphorylation of hexose monophosphate or glucose. When arsenite is added, to prevent oxidation of any pyruvate which may be formed, the ratio of atoms of phosphorus esterified to molecules of oxygen taken up is half or less of that obtained (without arsenite) with pyruvate-fumarate. Hence catalytic action of C_4 dicarboxylic acids by itself can account for no more than half of the phosphorylation connected with pyruvate oxidation.

BIBLIOGRAPHY

1. Ochoa, S., *Nature,* **145,** 747 (1940); **146,** 267 (1940).
2. Banga, I., Ochoa, S., and Peters, R A , *Biochem J.,* **33,** 1109 (1939).
3. Ochoa, S., *Nature,* **144,** 834 (1939).
4 Banga, I., Ochoa, S., and Peters, R. A., *Biochem J* , **33,** 1980 (1939)
5 Lipmann, F., *Enzymologia,* **4,** 65 (1937); *Nature,* **143,** 281 (1939)
6. Kalckar, H., *Enzymologia,* **2,** 47 (1937), **5,** 365 (1939), **6,** 209 (1939).
7. Kalckar, H , *Biochem J* , **33,** 631 (1939).
8. Colowick, S. P , Welch, M. S., and Cori, C. F , *J Biol. Chem.,* **133,** 359, 641 (1940)
9 Clift, F. P , and Cook, R P., *Biochem. J* , **26,** 1788 (1932)
10. Roe, J. H , *J. Biol. Chem* , **107,** 15 (1934)
11. Smythe, C. V , *J. Biol Chem.,* **118,** 619 (1937).
12. Vercellone, A , and Neuberg, C., *Biochem. Z* , **280,** 161 (1935).
13. Meyerhof, O , and Lohmann, K., *Biochem. Z* , **271,** 89 (1934).
14. Kiessling, W., *Ber. chem. Ges* , **67,** 869 (1934)
15. Reis, J , *Enzymologia,* **2,** 110 (1937).
16. Rapoport, S , *Biochem Z.,* **291,** 429 (1937)
17. Geiger, A , *Biochem J* , **34,** 465 (1940).
18. Needham, D. M., and Pillai, R K., *Biochem J.,* **31,** 1837 (1937).
19. Meyerhof, O., Ohlmeyer, P., and Mohle, W , *Biochem Z.,* **297,** 90 (1938).
20 Meyerhof, O , *Bull Soc. chim. biol.,* **20,** 1335 (1938).
21. Ostern, P., Guthke, J. A., and Terszakoweć, J , *Z. physiol. Chem.,* **243,** 9 (1936).
22. Meyerhof, O., *Naturwissenschaften,* **23,** 850 (1935).
23. Case, E. M., *Biochem. J* , **23,** 210 (1929).
24. Adler, E., von Euler, H., and Günther, G., *Skand Arch. Physiol.,* **80,** 1 (1938).
25. Krebs, H. A , and Johnson, W. A., *Biochem. J* , **31,** 645 (1937).
26. Lennerstrand, A , *Naturwissenschaften,* **25,** 347 (1937)
27. Needham, D. M., and Lu, G. D., *Biochem. J.,* **32,** 2040 (1938).
28. Warburg, O , and Christian, W , *Biochem Z.,* **303,** 40 (1939).
29. Negelein, E , and Bromel, H , *Biochem. Z.,* **303,** 132 (1939).
30. Lipmann, F., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7,** 248 (1939)
31 Lipmann, F., *J. Biol Chem* , **134,** 463 (1940)
32. Long, C., *Biochem J.,* **32,** 1711 (1938).
33. Ochoa, S , Peters, R. A., and Stocken, L. A., *Nature,* **144,** 750 (1939).
34. Belitzer, V. A., and Tsibakowa, E. T., *Biokhimiya,* **4,** 516 (1939)
35 Mawson, C. A., *J. Physiol.,* **78,** 295 (1933).
36. Belitzer, V. A., *Enzymologia,* **6,** 1 (1939).

A FLAVOPROTEIN FROM YEAST

By D. E. GREEN,* W. E. KNOX, and P. K. STUMPF

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

With a Note by J. L. Oncley

In the course of the isolation of carboxylase from top brewers' yeast (1) we observed that crude preparations of the enzyme were characterized by a deep yellow color. Examination disclosed that some flavoprotein was mainly responsible for the yellow color. The present communication deals with the purification and properties of this chromoprotein.

The isolation procedure leads to a preparation in which the flavoprotein component accounts for about 60 per cent of the total sedimenting material. All the color, however, is associated with the flavoprotein component. The ultracentrifuge data reported in the accompanying note by Oncley suggest that the flavoprotein has a molecular weight of from 50,000 to 70,000, assuming a frictional ratio of from 1.0 to 1.2. Another colored substance in addition to flavin is found combined with this protein, though as yet nothing is known of its chemical nature.

Method of Isolation

Brewers' ale yeast is washed with 5 volumes of water and dried at room temperature in a brisk current of air (within 24 hours). 500 gm. of finely ground, dried yeast are stirred slowly into 1500 cc. of water heated to 42°. The suspension is incubated at 38° for 1 hour, mixed with 2000 cc. of water, and centrifuged.

The opalescent supernatant fluid (2620 cc.) is mixed in order with 400 cc. of 0.5 M disodium phosphate and 200 cc. of M calcium acetate. After the precipitate is centrifuged off, a clear yellow solution is obtained (2900 cc.). 28 gm. of ammonium sulfate

* Beit Memorial Research Fellow.

are added for each 100 cc. of solution. Centrifuging for at least 1 hour is necessary to separate the precipitate effectively. The precipitate is discarded and 12 gm. of ammonium sulfate are added for each 100 cc. of supernatant fluid (volume about 3200 cc.). The precipitate (250 cc.) is filtered off, and dissolved by addition of 500 cc. of water. The precipitation of the flavoprotein is repeated by addition of 200 gm. of ammonium sulfate. The precipitate is dissolved in water in a final volume of 300 cc. The solution is filtered and dialyzed for 3 hours against running tap water.

The dialyzed solution (490 cc.) is mixed with 75 cc. of alumina C-γ gel (27 mg. dry weight per cc.). The gel is centrifuged off and discarded. A further addition of 250 cc. of the alumina gel is made. The gel is washed with 5 volumes of water and eluted three times with 40 cc. portions of 0.5 M disodium phosphate.

The combined eluates (volume 120 cc.) are then submitted to the first ammonium sulfate fractionation. Four fractions are obtained by centrifuging off the precipitates formed after addition of 0.35, 0.56, 0.83, and 1.38 volumes respectively of saturated ammonium sulfate. The first and last fractions are discarded; the second and third are combined (volume 53 cc.), and the solution is dialyzed for 3 hours.

The second alumina C-γ adsorption is now carried out. 25 cc. of the alumina gel suffice to remove the flavoprotein quantitatively, leaving a considerable amount of colorless impurity behind. The gel is washed with water and eluted three times with 25 cc. portions of 0.5 M disodium phosphate. The eluates are combined and the flavoprotein is precipitated by addition of 40 gm. of ammonium sulfate for each 100 cc. of solution. The precipitate is dissolved in about 25 cc. of water, and the solution is clarified by filtration.

Saturated ammonium sulfate solution is added slowly until the first turbidity forms. The precipitate is discarded. More ammonium sulfate solution is added until the bulk of the protein precipitates. The precipitate is centrifuged off, and dissolved in the minimum volume of water.

The yield from 500 gm. of dried yeast at this level of purity is about 40 mg. of flavoprotein containing 344 γ of bound flavin-adenine dinucleotide. The isolation involves roughly a 500-fold increase in purity.

Properties of the Flavoprotein

Dilute solutions of top yeast flavoprotein (<1 mg. per cc.) appear yellow-green in color. More concentrated solutions (>2 mg. per cc.) appear brownish yellow and finally brownish red. On addition of $Na_2S_2O_4$ the color is bleached to the extent of 46 per cent. Shaking with air restores the original color. This process can be repeated many times. When the flavoprotein solution is boiled, the protein is denatured and the flavin group is split off. The protein-free filtrate has the typical lemon-yellow

Wavelength in mμ
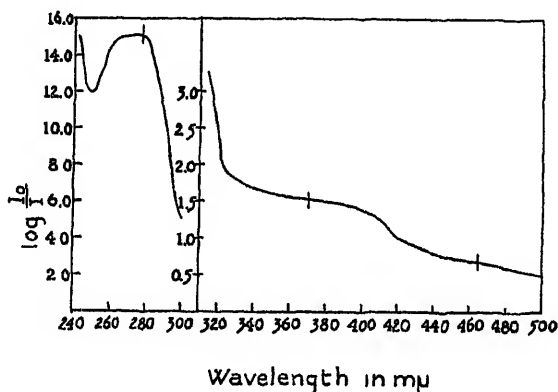
Fig 1. The absorption spectrum of yeast flavoprotein preparation at the 0.86 per cent flavin phosphate stage. The curves were obtained on a Hilger Spekker photometer. The solution contained 1.59 mg. per cc., $d = 1$ cm.

color and green fluorescence of flavin. Addition of $Na_2S_2O_4$ now bleaches the color completely.

The absorption spectrum of the flavoprotein preparation at the highest level of purity reached is shown in Fig. 1. There are three maxima at 278, 370, and 465 mμ respectively. The extinction ratio $\epsilon_{278}/\epsilon_{450} = 20$ and $\epsilon_{278}/\epsilon_{465} = 22$. The absorption spectrum differs from that of a typical flavoprotein in two respects. (1) The bands at 370 mμ and 465 mμ do not show up as definite peaks, and (2) the 370 mμ band is higher than the 465 mμ band. These discrepancies argue the presence of another colored constituent in addition to flavin, and indeed it can easily be demonstrated that the light absorption of the flavoprotein in the visible region

of the spectrum is only partly referable to flavin. On addition of $Na_2S_2O_4$ to the flavoprotein solution the color is bleached to the extent of 37 per cent at 450 mμ and 46 per cent at 465 mμ (*cf.* Fig. 2). If all the color were due to flavin, bleaching would have been complete. It is significant that in plotting the difference in light absorption between oxidized and reduced flavoprotein
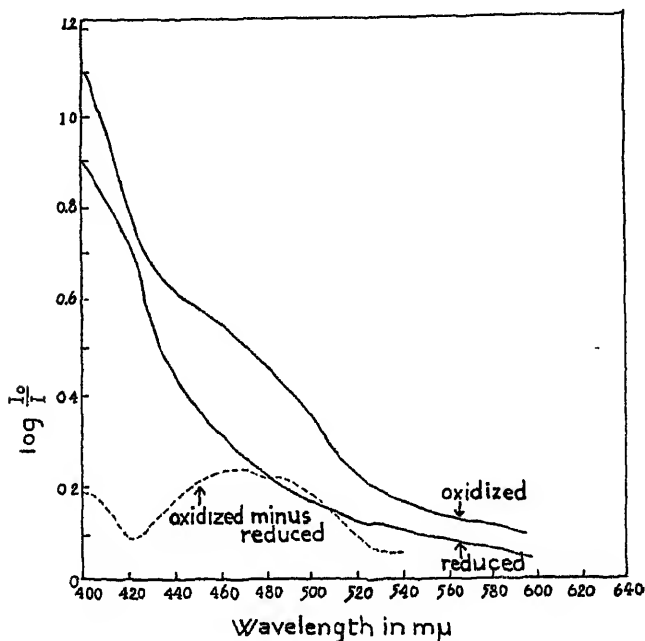
FIG. 2. The visible absorption spectrum of oxidized and reduced yeast flavoprotein preparation. The curves were obtained on a Hardy automatic spectrophotometer.

against wave-length a typical flavin spectrum with a peak at 465 mμ is obtained.

When a solution of the flavoprotein is boiled or acidified ($<$ pH 4), the flavin group is split off and the protein is denatured. The insoluble protein can, however, be dissolved in dilute sodium hydroxide. The absorption spectrum of the flavin-free denatured protein is identical with that of the native flavoprotein reduced with $Na_2S_2O_4$. Furthermore, addition of $Na_2S_2O_4$ has no influence on

the absorption spectrum. It is thus clear that the non-flavin colored group is not reducible by $Na_2S_2O_4$ and, unlike flavin, is not detached from the protein when the flavoprotein is denatured by either boiling or acidification.

The estimate that flavin accounts for 46 per cent of the total light absorption at 465 mμ is based on two different lines of evidence: (1) the decrease in absorption at 465 mμ after reduction with $Na_2S_2O_4$, and (2) the yield of flavin liberated from the denatured flavoprotein. The estimates by these two different methods are identical within the limits of experimental error. For example, a solution of the flavoprotein showed an extinction of 0.46 at 465 mμ. Assuming a β value of 2.4×10^7 for the absorption of flavin at 465 mμ (2), and assuming that flavin accounts for 46 per cent of the absorption at that wave-length, the solution should have contained 9.25 γ of flavin phosphate per cc. The flavoprotein was denatured by acidification with glacial acetic acid, and the insoluble protein removed by centrifugation. Colorimetric and spectrophotometric estimations of flavin in the supernatant fluid showed 8.8 γ of flavin phosphate per cc. of the original solution; i.e., 95 per cent of theory.

Thus far we have not discussed the form in which flavin occurs. All known flavoproteins, with one exception, contain flavin in the form of flavin-adenine dinucleotide. The presence and amount of the dinucleotide can be determined manometrically in the d-amino acid oxidase test (3). Estimation by this test showed that all the flavin liberated by acid denaturation of the flavoprotein was quantitatively accounted for as dinucleotide (98 per cent recovery).

At the highest purity level attained in the purification of the flavoprotein ($\epsilon_{278}/\epsilon_{450} = 20$), the percentage of flavin phosphate was 0.86. The ultracentrifuge data (cf. accompanying note by Oncley) indicates that the flavoprotein accounts for about 60 per cent of the total sedimenting protein present in the preparation. The homogeneous protein should contain, therefore, at least 1.4 per cent of flavin phosphate. This would be slightly more than twice the percentage found for typical flavoproteins.

Tests for Catalytic Activity

As yet no catalytic function has been found for the flavoprotein. The following substrates have been found negative in aerobic and

anaerobic test systems: aldehyde, alcohol, hypoxanthine, dihydrocoenzyme I, *dl*-alanine, glucose, glyceraldehyde, butyrate, isoamylamine, adrenalin, α-glycerophosphate, glycerol, formate, *d*-glutamate, histamine, *d*-aspartate, lactate, pyruvate, α-hydroxyglutarate, succinate, fumarate, oxaloacetate, and α-ketoglutarate. Crude preparations of the flavoprotein show some activity in catalyzing the oxidation of dihydrocoenzyme I However, with further purification this activity disappears.

Relationship to Other Flavoproteins

The flavoprotein described above is not identical with any of the flavoproteins described in the literature. It bears, however, some structural resemblances to the liver aldehyde oxidase (4) and milk xanthine-aldehyde-dihydrocoenzyme I oxidase (5). These three flavoproteins have the following properties in common: (1) brownish color in concentrated solution; (2) flavin accounting for 33 to 37 per cent of the total absorption at 450 mμ; (3) a non-flavin colored group with general absorption in the visible region. At the moment it would be premature to consider the non-flavin colored group as identical in the three flavoproteins, though the available evidence is in favor of this view. This group has the same type of spectrum in the three cases. Furthermore, it is always found in very firm combination with the protein. Denaturation whether by acid or heat does not liberate the non-flavin colored group from the protein.

BIBLIOGRAPHY

1. Green, D. E., Herbert, D., and Subrahmanyan, V., *J. Biol. Chem.*, **135**, 795 (1940).
2. Theorell, H , *Biochem. Z*, **278**, 263 (1935).

3. Corran, H S , Green, D E , and Straub, F B , *Biochem J* , **33**, 793 (1939)
4. Gordon, A H , Green, D E , and Subrahmanyan, V , *Biochem J* , **34**, 1108 (1940)
5. Corran, H S , Dewan, J G , Gordon, A H , and Green, D E , *Biochem. J* , **33**, 1694 (1939)   Ball, E G , *J Biol. Chem.*, **128**, 51 (1939)

Note on Ultracentrifuge Analysis

By J L Oncley

(From the Department of Physical Chemistry, Harvard Medical School, Boston)

The ultracentrifugal analysis was conducted upon two solutions, the first estimated to contain about 1 per cent of protein, and the second about 2 per cent.   These solutions had been exhaustively dialyzed against 0.2 M potassium chloride.   The ultracentrifuge, of the air-driven type,[1] was equipped with a modified Philpot-

Figs A and B Sedimentation diagrams of yeast flavoprotein   Taken 60 minutes after reaching full speed (54,000 R P M)   Fig A, with yellow light, Fig B, with blue light   The meniscus is on the right, and sedimentation occurs toward the left.

schlieren system,[2] and was run at a speed of 54,000 R.P.M. with a cell 15 mm. high and a solution thickness of 10 mm.   Fig A shows the sedimentation diagram obtained with yellow light (Wratten Filter 22 and Wratten M plate), and Fig. B with blue and violet light (unfiltered, but with an Eastman Contrast-Process plate).   These diagrams were obtained 60 minutes after full speed was reached

Analysis of the results revealed that only about one-half of the material contributing to the refractive increment was observably

[1] Bauer, J H , and Pickels, E G , *J Exp Med* , **65**, 565 (1937)   Pickels, E. G , *Rev Scient Instruments*, **9**, 358 (1938)
[2] Philpot, J. St L , *Nature*, **141**, 283 (1938)

sedimenting material. Of this sedimenting material, the main component was present in amounts of about 60 per cent, had a sedimentation constant of about 4.5×10^{-13} ($s_{20,w}$), and was responsible for nearly all of the light absorption. A faster moving component ($s_{20,w}$ about 9×10^{-13}) was present to the extent of about 20 per cent, and a slower moving component ($s_{20,w}$ between 2 and 3×10^{-13}) was present to the extent of about 20 per cent. No observable light absorption between 350 and 500 mμ wave-length was associated with these two components (Fig. B).

LETTERS TO THE EDITORS

A NEW COLOR REACTION FOR THE PHENOLIC STEROIDS (NATURALLY OCCURRING ESTROGENS)

Sirs:

An interesting substance having specific properties results from the coupling of estrone, estriol, or estradiol with phthalic anhydride. The basis is the well known work of Baeyer[1] in which he produced phenolphthalein from phenol and phthalic anhydride. Advantage is taken of the possession of a phenolic hydroxyl group by the three hormones mentioned.

The procedure of a typical experiment is as follows: 1 cc. of a chloroform solution of 40 γ of estrone is pipetted into an ordinary Pyrex test-tube, 1 cc. of 1 per cent phthalic anhydride in 95 per cent ethyl alcohol is added, and the mixture placed on a steam bath until dry. About 0 2 cc. of anhydrous stannic chloride are added from a bulb pipette, and the tube is rotated so that the stannic chloride moistens all of the dry material. It is then placed in an oven at 116–120° for 1½ hours, after which it is transferred to a desiccator to cool. A dark red to reddish brown residue results. Upon addition of 5 cc. of chloroform with thorough stirring, this residue dissolves to produce a solution having a brilliant deep pink color with greenish yellow fluorescence.

This color solution has an absorption band[2] at 538.6 mμ. The fluorescence band has its maximum at 557 mμ. The fluorescence appears also in ultraviolet light. However, in ultraviolet light there is more rapid fading than in visible light.

Estrone,[3] estradiol, and estriol, all of which have the phenolic

[1] Baeyer, A , *Ann. Chem* , **202**, 36 (1880).

[2] I am indebted to Dr. Erich Schneider for the spectroscopic data and to Dr Liese Lewis for the fluorimetric tests.

[3] I wish to acknowledge the courtesy of Dr. Erwin Schwenk of the Schering Corporation for the samples of estrone, estradiol, androsterone, dehydroandrosterone, testosterone, and progesterone and that of Dr. Oliver Kamm of Parke, Davis and Company for samples of theelin and theelol.

hydroxyl group, yield colored products having the same optical properties. Cholesterol, testosterone, androsterone, dehydroandrosterone, and progesterone, which do not possess this grouping, do not produce this phthalein. ·Although any substance containing a phenol hydroxide group will form a phthalein in the same way, none of those tested thus far (phenol, tricresol, α-naphthol, thymol, β-hydroxybenzoic acid, adrenalin, l-tyrosine, resorcinol, and pyrogallic acid) yields compounds having similar properties. These properties will, it is hoped, afford means of adapting the test to quantitative procedures.

Preliminary tests indicate that quantitative results may be obtained either colorimetrically or fluorimetrically but the optimum conditions for producing the color have not yet been ascertained, nor has any attempt been made as yet to apply this test to extracts of urine, blood, or tissues.

The test is extremely delicate. By using appropriate amounts of reagents, as little as 0.25 γ of estrone may be detected.

Department of Physiology and Biochemistry   Israël S. Kleiner
   New York Medical College, Flower and
     Fifth Avenue Hospitals
   New York

A PROCEDURE FOR THE DECOLORIZATION OF ACID DIGESTION MIXTURES FOR THE DETERMINATION OF NICOTINIC ACID

Sirs:

The following simple procedure yields clear, almost colorless solutions from acid digestion mixtures of whole blood, dried blood, urine, and tissues. The precipitation of colored material is accomplished in the digestion tube and the mixture is cleared by centrifugation. The method possesses the advantage of simplicity and permits the complete recovery of added nicotinic acid.

5 cc. of urine or blood or 5 gm. of tissue are transferred to a calibrated, heavy walled ignition tube such as is used in the determination of non-protein nitrogen. Exactly 5 cc. of 8 N HCl are added and the contents are given a preliminary mixing by gently rotating the tube back and forth vertically between the palms. It is often impossible to mix samples immediately after adding the HCl. However, it is accomplished if the tube is shaken after the sample has been digested for a few minutes in a bath of boiling water. The tube is kept covered during the digestion. In the case of blood and tissue, the contents of the tube are mixed several times during the course of the digestion. Urine is digested for 30 minutes in a bath of boiling water; blood and tissue for 60 minutes. The tube and contents are then cooled.

2 cc. of a solution containing 800 gm. of $Zn(SO_4) \cdot 7H_2O$ per liter are now added. Exactly 5 cc. of NaOH solution are added drop by drop. The hydroxide solution is prepared of such strength that 10.1 cc. are required to produce a faint pink color to phenolphthalein when added to exactly 5 cc. of the 8 N HCl, 2 cc. of the $ZnSO_4$ solution, and approximately 25 cc. of water. The contents are kept well mixed by means of a long, thin, footed glass rod, and are cooled under cold running water, after which the precipitation of zinc is completed by the addition of exactly 5 cc. more of the alkali. The mixture is again cooled, if necessary. 1 drop of caprylic alcohol is added, and the volume is adjusted to the 35 cc. mark. The tube is now closed by means of a rubber stopper and

the contents are well mixed. The tube should stand for at least 10 minutes, with occasional mixing of the contents, to assure complete diffusion of the nicotinic acid from the residue and more efficient adsorption of the color by the zinc hydroxide. The precipitate is separated from the solution by means of centrifugation. The reaction of the supernatant fluid should be colorless to phenolphthalein and alkaline to phenol red.

For those materials which contain very large quantities of yellow substances, or which yield unusually large amounts of dark pigments, the hydrolysis in the presence of stannous chloride may prove useful. Stannous chloride bleaches many colored compounds and almost entirely prevents browning during the acid hydrolysis. However, the recovery of added nicotinic acid may be somewhat low. Therefore, the procedure should be carefully controlled, and parallel runs should be made with added quantities of nicotinic acid.

Abbott Foundation for Medical Research   THEODORE E FRIEDEMANN
  Northwestern University Medical School   CLIFFORD J. BARBORKA
  Chicago

COAGULATION OF FIBRINOGEN BY SIMPLE ORGANIC SUBSTANCES AS A MODEL OF THROMBIN ACTION*

Sirs:

The addition of ninhydrin (triketohydrindene hydrate) to fibrinogen solutions or plasma has been found to produce typical fibrin clots. This reaction requires neither calcium nor thromboplastic factor and appears to parallel the action of thrombin in a remarkable manner. The coagulation time is dependent on the concentrations of both the fibrinogen and the ninhydrin. If the

TABLE I

Ninhydrin, *mg.*	0 88	0 44	0.22	0 11	0.06	0
Clotting time, *min.*	4	6	10	16	27	>120

Each tube contained 0.2 cc. of fibrinogen solution and 0.03 cc. of ninhydrin solution.

TABLE II

Clotting agent	Volume of reaction mixture	Fibrinogen N used	Fibrin N recovered	Fibrin analyses*	
				N	S
	cc.	*mg.*	*mg.*	*per cent*	*per cent*
Thrombin	20.5	18.3	16.0	15.4	0.98
Ninhydrin (54 mg.).	22	21.6	18.6	14.6	1.07
" (177.6 mg) . ..	35	22 8	19.1	15.0	1 02

* Calculated for preparations free of moisture and ash.

fibrinogen concentration is kept constant, a smooth curve is obtained when the coagulation time is plotted against ninhydrin concentration. A typical experiment is reproduced in Table I. The formation of fibrin from fibrinogen is not accompanied by coloration, although a slight blue tint appears when the clotted

mixture is heated or stored for some time. No coagulation of milk caseinogen is observed under these conditions.

A similar, although much less pronounced, clotting effect is obtained with another ketone, alloxan, and with salicylaldehyde. The following compounds were without effect in comparable concentrations: formaldehyde, acetone, pyruvic acid, urea, allantoin, isatin, methylene blue, potassium ferricyanide. Two compounds having vitamin K activity, the sparingly water-soluble 2-methyl-1,4-naphthoquinone and the sodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid, were likewise inactive. The reactions were carried out at 30° and at pH 6.9 to 7.2.

An attempt is being made to study the reactions involved, with a view to the elucidation of the mechanisms concerned in the physiological conversion of fibrinogen to fibrin. Analyses of fibrin samples obtained from horse fibrinogen by means of thrombin and ninhydrin respectively (Table II) indicate that compound formation between the latter reagent and the protein occurs to only a slight extent, if at all.

Departments of Biochemistry and Surgery ERWIN CHARGAFF
  College of Physicians and Surgeons MORRIS ZIFF
  Columbia University
  New York

THE ACTIVATION OF INTESTINAL PEPTIDASES BY MANGANESE

Sirs:

From the work of Berger and Johnson[1] it is known that the erepsin obtained from hog intestinal mucosa is activated by magnesium or manganese, or manganese and cysteine, and that the activated erepsin hydrolyzes both the *l* and the *d* form of leucylglycine.[2] It has now been found that the intestinal enzyme which splits *l*-leucylglycine is a metal-protein compound. During the purification of the protein component most of the metal was removed and simultaneously the activity in the absence and in the presence of added manganese dropped from equality to a ratio of 1:50. The combination of the protein with the activator metal is a time reaction, the reaction rate and total activity depending upon the concentration of metal. At pH 7.9 and 40°, a solution containing per cc. 50 γ of protein nitrogen and 0.01 mM $MnSO_4$ required more than 4 hours to attain its maximal activity. The metal-free protein is, at this temperature and pH, much more stable than is the Mn-protein compound.

Our enzyme preparations were colorless and water-clear solutions. After reaction with manganese, the enzyme hydrolyzed *l*-leucylglycine and *l*-leucylglycylglycine with a proteolytic coefficient[3] of 2.2 to 2.3; *i.e.*, in a reaction mixture containing 2.5 γ of protein nitrogen per cc. of the enzyme, *l*-leucylglycine was hydrolyzed more than 50 per cent in 1 hour (substrate = 0.05 mM per cc., 40°, pH 7.9).

Most of the activity of the crude extract of mucosa toward glycylglycine and *l*-alanylglycine was removed during the isolation of the enzyme which hydrolyzes *l*-leucylglycine. This shows that

[1] Berger, J., and Johnson, M. J., *J. Biol. Chem.*, **130**, 641 (1939).

[2] Berger, J., Johnson, M. J., and Baumann, C. A., *J Biol. Chem.*, **137**, 389 (1941).

[3] Irving, G. W., Jr., Fruton, J. S , and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).

the enzyme that hydrolyzes *l*-leucylglycine is different from the enzyme or enzymes that hydrolyze the other two peptides. Moreover, during the purification the ratio of the velocity constants for the Mn-activated hydrolysis of *d*-leucylglycine and *l*-leucylglycine decreased from 1/50 to 1/10,000. It is apparent that the two antipodes are hydrolyzed by distinct enzymes.

Since the intestinal enzymes that hydrolyze other peptides such as glycylglycine and prolylglycine are also activated by Mn,[1,4] it becomes clear that there are in intestinal mucosa several metal-containing proteases. The fact that the action of several peptidases is inhibited by HCN and H_2S[5] may be taken as indicating also that these enzymes are metal-protein compounds. The presence of metal-containing peptidases in anaerobic bacteria has been demonstrated by Maschmann.[6]

Laboratories of The Rockefeller   EMIL L SMITH
    Institute for Medical Research   MAX BERGMANN
    New York

[4] Berger, J., and Johnson, M. J , *J. Biol Chem.*, **133**, 639 (1940).

[5] Grassmann, W., and Dyckerhoff, H., *Z. physiol Chem.*, **179**, 41 (1928). Bergmann, M , Zervas, L., Fruton, J. S., Schneider, F., and Schleich, H., *J. Biol. Chem* , **109**, 325 (1935). Bergmann, M , and Fruton, J. S., *J. Biol. Chem.*, **117**, 189 (1937)

[6] Maschmann, E., *Biochem. Z* , **302**, 332 (1939).